THE EFFECT OF TOBACCO SMOKE ON RENEWAL MECHANISMS IN AIRWAY EPITHELIUM AND CHANGES INDUCED BY N-ACETYLCYSTEINE

A Thesis Presented for the Degree of

Doctor of Philosophy

by

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Anger and tenderness: my selves.
And now I can believe they breathe in me
as angels, not polarities.
Anger and tenderness: the spider's genius
to spin and weave in the same action
from her own body, anywhere -
even from a broken web.

Adrienne Rich, 1978.

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ABSTRACT

Hypersecretion is a feature of chronic bronchitis and other airway diseases, often with ensuing complications of infection, failure to clear secretions, airflow obstruction and progressive disability leading to premature death. The histological counterpart is an increase in mucus-secreting tissue: ie. hyperplasia of mucous cells in the surface epithelium and hypertrophy of submucosal glands. The former change, associated with an extension of mucous cells into peripheral airways from which mucus is difficult to clear by cough, is of particular functional significance. In the studies presented herein the laboratory rat, free of respiratory infection, has been used to study the pathogenesis of epithelial mucous cell hyperplasia induced by 2 weeks exposure to an atmosphere of tobacco smoke (TS). Morphological changes have been studied in 1 m plastic-embedded sections of main bronchus in which 7 distinct cell types were quantified. Proliferative indices for each cell type were assessed quantitatively by autoradiography of tissue sections following intraperitoneal injection of tritiated thymidine (^{3}H-T) given 1 hour prior to sacrifice of unexposed rats. The results were compared with those from rats killed on days 1, 3, 7, 10 or 14 of TS-exposure. Putative pathways of differentiation, metaplasia and maturation are indicated by the change with time in the distribution of autoradiographic grains between the distinct cell types following a single pulse of ^{3}H-T given only at the beginning of the experiment. The proliferating cell types in normal, unirritated epithelium are basal, which form 71% of the total labelled cell population, serous (17%), and indeterminate (9%); mucous and ciliated cells are maintained by differentiation of serous and basal

cells. The response to TS exposure is complex and dependent on the exposure conditions; it comprises a combination of a proliferative response, from either mucous cells or both basal and mucous cells, mucous metaplasia of serous cells and accelerated differentiation whereby the production of ciliated cells is increased. In comparison with the normal response, N-acetylcysteine (a drug with known mucolytic properties), given as a 1% solution in drinking water, modifies the proliferative response to TS, in particular that of basal cells, by delaying, reducing but prolonging it. The drug also restores normal pathways of differentiation. These findings suggest that modulation of the proliferative and differentiation responses is possible and may be an approach worthy of further investigation with a view to therapy of hypersecretory diseases.

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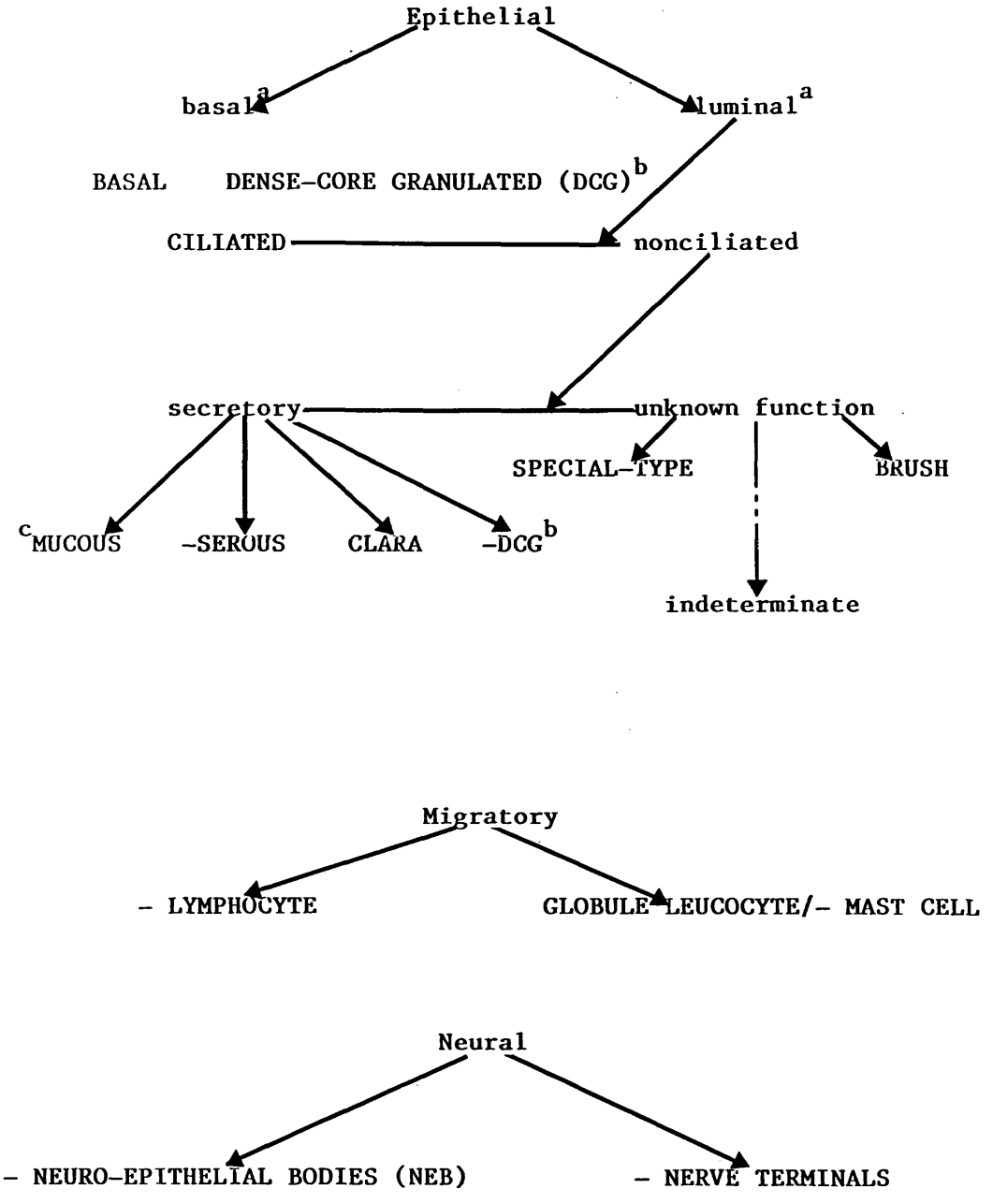
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CHAPTER 1 – INTRODUCTION: LITERATURE REVIEW

Chronic bronchitis, cystic fibrosis and often asthma are diseases in which hypersecretion and the resultant sputum production are major features (Wood et al., 1976; Fletcher and Pride, 1984; Anderson, 1986). Mucous cell hyperplasia is a hallmark of the hypersecretory state (Reid, 1954; de Haller and Reid, 1964; Dunnill, 1969; Crofton and Douglas, 1981). As the pathogenesis of mucous hyperplasia is still unclear, several experimental animal models have been constructed to investigate possible mechanisms. The models involved exposure of a variety of animal species, in particular rodents, to irritants such as sulphar dioxide (SO_2) (Reid, 1963; Lamb and Reid, 1968), tobacco smoke (TS) (see Jeffery and Reid, 1981) and neuromimitecs such as isoprenaline and pilocarpine (see Jones and Reid, 1979). Data from these studies demonstrated that mucous cell hyperplasia occurs in response both to irritants and drug exposure and raised fundamental questions about the mechanisms of the response. Do the newly-occurring mucous cells arise (i) from 'metaplasia' of existing cells not of a mucous phenotype, (ii) division of the small number of existing mucous cells or (iii) a combination of these two pathways?

The following review of the literature will set the scene for subsequent description of my investigation of these questions. Historical and recent information concerning the processes of proliferation and differentiation in airway epithelium will be surveyed; in addition, some of the intrinsic and extrinsic factors which influence these processes, particularly tobacco smoke exposure and the modulating effects of drugs, will be outlined.

FIGURE 1.1 MATURE AIRWAY EPITHELIUM - CELL TYPES



a. All cells may rest on the basal lamina but only some reach the surface (luminal).

b. Often basal, sometimes luminal.

c. In order to distinguish between cell types which occur in both surface epithelium and submucosal glands the terms "surface epithelial-" and "glandular-" should be prefixed. This is indicated where appropriate by a hyphen preceding the term.

1.1. EPITHELIAL RENEWAL

1.1.1. Airway Epithelial Cell Types

In 1881, Drasch described 4 cell types which comprise mammalian airway epithelium: ciliated, goblet, intermediate and basal cells. The infrequent observation of mitotic figures among these cells led to dispute about the occurrence of cell division in mammalian adult airway epithelium (Drasch, 1881; Waller and Bjorkman, 1882; Flemming, 1885; Bockendahl, 1885). Despite this controversy, the basal cell was proposed as the epithelial progenitor cell by Drasch, (1881) and Waller and Bjorkman, (1882).

More recently at least eight epithelial cell types have been recognized in the lining epithelium of conducting airways (ciliated, serous, mucous, Clara, brush cells, special type cells, dense core granule, and basal cells) (Fig. 1.1) and two cell types in the epithelium lining the alveoli (type I and II cells) (for reviews see Jeffery and Reid, 1975; Breeze and Wheeldon, 1977; Jeffery, 1983a; Jeffery and Corrin, 1984a). Mammalian tracheo-bronchial surface epithelium is pseudostratified, ciliated and columnar, reducing in thickness and degree of pseudostratification with airway generation, until the epithelium is simple columnar at the level of the small bronchioles (Miller, 1932). In normal human epithelium, two or more rows of basally situated cells are often observed (McDowell et al., 1978), whereas

in animals airway epithelium has one basal and one superficial layer of nuclei and all cells reach the basement membrane. Fig.1.1. shows epithelial cell types distinguished according to position (basal or lumenal), presence of cilia and secretory granules.

Quantitative investigation into cell division in the respiratory tract began in 1951 concentrating on the distal (ie respiratory) portion of the lung (reviewed by Bertalanffy, 1964 a, b; Kauffman, 1980; Masse et al., 1981). Studies of the lining epithelium of conducting airways soon followed (Leblond and Walker, 1956; Bjork, 1957; Bjork and Harkonen, 1960; Spencer and Shorter, 1962; Blenkinsopp, 1967; Wells, 1970; Bolduc and Reid, 1976; Boren and Paradise, 1978).

Early suggestions regarding the progenitor role of basal cells were confirmed by several workers, who demonstrated that the majority of epithelial cells in proximal airways arose from division and differentiation of basal cells (Condon, 1942; Rhodin, 1966; Blenkinsopp, 1967; Bindreiter et al., 1968; Bolduc and Reid, 1976). However, in peripheral bronchioles where basal cells are absent, the secretory Clara cell was found to be the progenitor: Clara cells divided in response to epithelial irritation and subsequently differentiated to form mature secretory and ciliated cells (Evans, et al., 1976a, b, c; 1977; 1978a, b). In the alveolus the type II cell has been shown to be the progenitor cell (Evans et al., 1973, 1975; Castleman et al., 1980).

There are three types of secretory cells, distinguished by the type of secretory granule they contain and the airway level at

which they occur: serous and mucous (ie. goblet) cells normally occur in the proximal airways only (Jeffery and Reid, 1975), while Clara cells are found distally limited to the small airways of most species (Plopper et al., 1980; Pack et al., 1981). Observation and quantitation of dividing serous and mucous cells in a number of experimental models of injury (Boren and Paradise, 1978; Ayers and Jeffery, 1982; Keenan et al., 1982 a,b,c; Keenan et al., 1983; McDowell et al., 1984 a,b) has led to the recent proposal that the secretory cell plays the major generative role in respiratory epithelium (McDowell and Trump, 1983; Evans et al., 1986).

Therefore the surface epithelial cells which have a capacity to divide are the basal, serous, mucous, Clara and alveolar type II cells: the number of each which contributes to the pool of dividing cells varies with airway level and species.

1.1.2. Proliferation

Most cell populations are made up of both proliferating and non-proliferating compartments (ie. cells in G_o, and many mature functional cells). Each proliferating cell passes through the cell cycle (see Section 1.1.2.1) which results in division by mitosis. Cell differentiation is defined herein as the development by a non-specialized cell of definable functional characteristics, a process which may or may not be preceded by division and is sometimes associated with a decreasing capacity for division (Leblond, 1964). In the following discussion of renewal processes and their variation in untreated respiratory epithelium, information about proliferation and differentiation has been

separated for simplicity, although clearly both processes occur concurrently and interact with each other. Renewal in respiratory epithelium has previously been reviewed by Boren and Paradise (1978), Kauffman (1980), Bowden (1983) and Wright and Alison (1984).

1.1.2.1. Measurement: Most of the data about proliferation and its variability in respiratory epithelium has been obtained by measurement of four basic parameters: mitotic index, rate of entry into mitosis, turnover time and labelling index.

Quantitation of the number of cells in mitosis allows three of these indices to be determined: Mitotic Index (MI), is the number of cells in mitosis at any one time (expressed as a ratio of the total number of cells counted). Rate of Entry into Mitosis (REM) is calculated from the accumulation of mitoses during time (t) after administration of stathmokinetic agents such as colchicine (Eigsti and Dustin, 1955; Stevens-Hooper, 1961; Tannock, 1967) and the vinca alkaloids vinblastine and vincristine (Tannock, 1967; Clark, 1971). The accumulated mitotic count is then divided by t to give REM, which represents a measure of the birth-rate of new cells. Turnover Time (TT), can be determined from REM if the total number of cells present is known (LeBlond and Walker, 1956; Stevans-Hooper, 1961; Bertalanaffy, 1964a, b; Lamerton and Steel, 1968; Aherne et al., 1977). Implicit in this determination is the assumption that all cells in the tissue are dividing and at the same rate. It should be noted, however, that the renewal rate of a tissue depends not only on the number of dividing cells (which *in vivo* is usually only a fraction of the population) but also on the

length of time taken for each cell to divide. When the two variables are unknown or altered the calculation of TT based on REM is inaccurate.

The cell cycle concept, proposing that dividing cells pass through a series of complex biochemical events comprising DNA synthesis (the S phase) and mitosis (M) separated by two gap phases (G_1 and G_2), was formulated in 1953 (Howard and Pelc, 1953). When either of the DNA precursors thymidine (T), or iododeoxyuridine (IUdR), labelled with tritium (^3H) or carbon-14 (^{14}C) (low energy particle emitters) is made available to a population of cells which are 'cycling' those in the S phase incorporate it irreversibly into their DNA; the extent of the incorporation is dependent on the activity of the enzyme, thymidine kinase. Thereafter the cell is 'labelled' and can be visualized by autoradiography, (Rogers, 1979), enabling the proportion of cells synthesizing DNA, ie. the Labelling Index (LI) to be calculated (LI = number of cells labelled divided by the number of cells counted). In a population, where all the cells are cycling, the number of cells in any phase of the cycle, is directly proportional to the length of that phase. Thus as the time for DNA synthesis (t_S) is longer than the time for mitosis (t_M) (Lamerton and Steel, 1968; Aherne et al., 1977), the LI for a given population will be correspondingly higher than the MI.

The simple technique of pulse labelling with tritium- or carbon-labelled thymidine and other more complex methods (described in Section 1.4) can be used to obtain information about the length of cell cycle phases, the growth fraction and rates of cell birth

and loss, but scant information is as yet available about these parameters in the respiratory tract.

1.1.2.2. Data based on cell division. Mitotic Index (MI): dividing tracheal epithelial cells were first found in the latter part of the 19th century (Drasch, 1881; Bockendahl, 1885; Carleton, 1923-1924). The results of contemporary studies of MI and REM (rate of entry into mitosis) in the respiratory tract are summarized and tabulated in Table 1.1. To enable comparison, the MI data have all been expressed as: the number of cells entering mitosis per 1000 cells counted per colchicine hour (ie REM). The REM of airway and alveolar epithelia is indeed small, the calculated range for the respiratory tract being 0.14 - 3 mitoses per 1000 cells per hour. By contrast REM has been estimated for rat duodenal epithelium as 30 mitoses per 1000 cells per hour (Leblond and Stevans, 1948), and for oesophageal epithelium of 2 month old mice 11 mitoses per 1000 cells per hour (Blenkinsopp, 1969).

Turnover Time (TT): as has been pointed out, this parameter (based only on quantitation of mitoses) is of questionable value. With this in mind, it is informative to survey its measurement in respiratory epithelium. TT has been calculated for a variety of species, airway levels and ages in both sexes (Table 1.2). Several methods have been used: in the studies of Spencer and his colleagues (Spencer and Shorter, 1962; Shorter et al., 1964, 1966; Divertie et al., 1968), the time for each labelled cell type to migrate (ie. 'migration time') to the epithelial surface and disappear or 'slough', was observed and assumed to be the TT for distinct cell populations. However this estimate was made without

TABLE 1.2 a)

RESPIRATORY EPITHELIUM: MIGRATION/TURNOVER TIME

Airway Level	Animal	Sex	Age (wks) Weight (g)	Migration Time (days)	Reference
Trachea	mouse	♂♀	adult	2	Shorter et al., 1964
	mouse		adult	5-7	Shorter et al., 1964
Trachea	rat	♂♀	adult	6-7	Shorter et al., 1966
Bronchus					
- large	mouse	?	2.6	3 & 21	Spencer & Shorter, 1962
- small	mouse	?	2.6	7-10	Spencer & Shorter, 1962
Bronchus	rat	♂♀	adult	7-8	Shorter et al., 1966
Alveolus	mouse	?	4	7	Spencer & Shorter, 1962
	mouse	?	4	21	Spencer & Shorter, 1962
				Turnover Time (days)	
Trachea	rat	♂	adult		Blenkinsopp, 1967
	- S.D.*		200 g	67	Blenkinsopp, 1967
	- B.H.*		200 g	100	Blenkinsopp, 1967
	- B.H.*		330 g	111	Blenkinsopp, 1967
	rat				
	- C.D.*	♂	5	11-22	Wells, 1970
	- M.D.*		5	37-42	Wells, 1970
	rat	♂♀	5	22-40	Bolduc, 1976

TABLE 1.2 b)

RESPIRATORY EPITHELIUM: MIGRATION/TURNOVER TIME

Airway Level	Animal	Sex	Age (wks) Weight (g)	Turnover Time (days)	Reference
Trachea	rat	♂♀	7	40-60	Bolduc, 1976
	rat		15	120-180	Bolduc, 1976
Trachea	hamster	♂	5	73	Wells, 1970
Broncus - main	rat B.H.*	♂	330 g	126	Blenkinsopp, 1967
- small	rat B.H.*		330 g	167	Blenkinsopp, 1967
Bronchiole	rat B.H.*	♂	330 g	200	Blenkinsopp, 1967
Alveolus	mouse - A/Gr_b	♂	12	44	Simnett & Heppleston, 1966
	mouse - A/Gr_b	♀	12	35	Simnett & Heppleston, 1966
	mouse - A/Gr_b	♂	52	84	Simnett & Heppleston, 1966
	mouse - A/Gr_b	♀	52	35	Simnett & Heppleston, 1966

*S.D. = Sprague Dawley

*B.H. = Black Hooded

*C.D. = Conventionally Derived

*M.D. = Minimum Disease

TABLE 1.1

RESPIRATORY EPITHELIUM: RATE OF ENTRY INTO MITOSIS (REM*)

Airway Level	Animal	Sex	Age (wks)	REM*	Reference
Trachea	mouse	?	8	3	Bjork, 1957
	mouse	?	12	0.16	Bjork et al., 1960
	mouse	♂	8	1.7	Blenkinsopp, 1969
	rat	♂	5	2.6	Bolduc & Reid, 1976
	rat	♀	5	0.9	Bolduc & Reid, 1976
Trachea - Basal Cell	hamster	♂	9-13	0.25	Boren & Paradise, 1978
Trachea - Mucous Cell	hamster	♂	9-13	0.225	Boren & Paradise, 1978
Bronchi	mouse	?	8	0.3	Bjork, 1957
	mouse	?	12	0.15	Bjork et al., 1960
Alveolus	guinea pig	♂	adult	1.5	Bertalanffy, 1953
	rat	♂	adult	1.5	Bertalanffy, 1953
	mouse (Strain A)	♂	12	0.7	Simnett & Heppleston, 1966
	mouse	♀	12	1.6	Simnett & Heppleston, 1966

*REM = no. mitoses/1000 total cells counted/hr. mitotic arrest due to colchicine

accounting for differences between cell types with respect to t_M, t_S, or the contribution of each cell type to the growth fraction of each population. TT was also calculated from counts of either the mitotic or labelling indices (Simnett and Heppleston, 1966; Bolduc, 1976), but again no account was taken of differences in either t_S or the growth fraction. Blenkinsopp (1967) assumed a value for t_S of 8h and used his LI data, to calculate that the TT for different airway levels in 2 strains of rats of varying ages ranged from 67 to 111 days. Wells (1970), also calculated TT of rat tracheal epithelium using data obtained from pulse labelling with ^{3}H-T. With time, labelled basal cells migrated into the superficial differentiating layer, thus the ratio of labelled basal cells to total labelled cells in the epithelium decreased. From the rate of decrease of the ratio, the TT was calculated for 2 'grades' of 5 week old rats: 11-22 days, for animals suffering from chronic respiratory disease and 37-42 days for animals relatively clear of respiratory disease.

Labelling Index (LI): since ^{3}H-T is taken up during the S phase of the cell cycle, longer by an order of magnitude than the time for mitosis (Messier and Leblond, 1960; Cleaver, 1967; Aherne, 1977), a relatively larger proportion of the proliferating population is labelled and available for quantitation, than is seen in mitosis. The LI range for the respiratory tract as quoted in the literature is broad: 0.02 - 40 labelled cells per 1000 cells counted. This figure covers a variety of species and airway levels (Table 1.3). The wide range of these figures illustrates that both MI and LI vary with changing intrinsic aspects of the respiratory tract and changing extrinsic conditions; the effects of these

TABLE 1.3 a)

RESPIRATORY EPITHELIUM: LABELLING INDEX

Airway Level	Animal	Sex	Age (wks)	L.I. (Labelled cells/1000 cells counted)	Reference
Trachea	rat	♂	adult	4	Messier & Le Blond, 1960
	rat	♂♀	adult	5-12	Shorter et al., 1966
	rat: C.D.*	♂	5	20	Wells, 1970
	rat: M.D.*	♂	5	7	Wells, 1970
Trachea	mouse	♂	?	5-17	Meyerzum Gottesburg, 1963
	mouse	♂♀	adult	10-40	Shorter et al., 1964
	mouse	♂♀	adult	14	Divertie et al., 1968
Trachea	rat	♂	3.6	23	Donnelly et al., 1982
Trachea	monkey (bonnet)	♂	adult	7	Wilson et al., 1984
Trachea	hamster	♂	5	4	Wells, 1970
- basal cell	hamster	♂	adult	5	Harris et al., 1975
- mucous cell	hamster		adult	2	Harris et al., 1975
- basal cell	hamster	♂	adult	11	Boren & Paradise, 1978
- mucous cell	hamster		adult	4	Boren & Paradise, 1978
Bronchus	rat	?	adult	10	Schultze & Oehlert, 1960
	rat	♂♀	adult	2-4	Shorter et al., 1966
Bronchus (anterior mainstem)	monkey (bonnet)	♂	adult	8	Wilson et al., 1984
Bronchus (intrapulmonary)	rat	♂	4.3	16	Evans et al., 1986

TABLE 1.3 b)

RESPIRATORY EPITHELIUM: LABELLING INDEX

Airway Level	Animal	Sex	Age (wks)	L.I. (Labelled cells/1000 cells counted)	Reference
Bronchus - large	mouse	♂♀	adult	8	Shorter et al., 1964
- small	mouse		adult	4-10	Shorter et al., 1964
Bronchus - large	mouse	♂♀	adult	12	Divertie et al., 1968
- small	mouse		adult	2	Divertie et al., 1968
Bronchiole	human in vitro	?	adult	2-7	Kury et al., 1969
Alveolus	mouse	♂	12	7	Simnett & Heppleston, 1966
	mouse		52	4	Simnett & Heppleston, 1966
	mouse		78	4	Simnett & Heppleston, 1966

*C.D. = Conventionally Derived

*M.D. = Minimal Disease

factors will be discussed in Sections 1.1.2.4. and 1.1.2.5.

1.1.2.3. Data based on the cell cycle - Cell Cycle and Phase Times: there are few studies of respiratory epithelium which determine the length of the cell cycle (T_C) and its phases for distinct cell types. Results vary widely: colchicine-induced metaphase block and ^{3}H-T labelling in mice gave a value of 380 h for T_C of tracheal basal cells (Blenkinsopp, 1969) (Table 1.4). Using other techniques, T_C for hamster tracheal basal cells was found to be both 28 h and 159 $\pm$ 11 h, while the value for mucous cells was 25 h and 97 $\pm$ 4 h respectively (Boren and Paradise, 1976, 1978). Table 1.5 shows the findings for different phases of the cell cycle for the tracheal basal cell: t_S ranged from 6 - 12 h and t_{G1} from 12 - 144 h (Boren and Paradise, 1976; 1978). Comparison of t_S of mouse alveolar wall cells in males and females of 3 strains at 3 ages showed that: (i) t_S was longer in males than females (2 out of 3 strains); (ii) t_S became longer with age (1 strain only); and (iii) t_S varied between strains (Simnett and Hepplestone, 1966). Such comparisons for other species and airway generations have yet to be made. The reported values for t_{G2} are relatively constant and lie between 3 and 4 h for both basal and superficial (including mucous) cells. For t_M there are only two reports for the respiratory tract (trachea). Findings for both basal and superficial cells were similar and ranged from 12 to 36 min (Boren and Paradise, 1978). The reason for the variation was not made clear. In other tissues t_M has been reported as 1 - 2 h in mouse forestomach epithelium (Wolfsburg, 1964); 2.0 h in mouse ear epidermis (Pilgrim et al., 1966), and 1.7 h in adult (8 weeks old) rat liver (Post and Hoffman, 1964).

TABLE 1.4

RESPIRATORY EPITHELIUM: CELL CYCLE TIMES

Tracheal Cell Type	Animal	Sex	Age (wks)	Method of Measurement	Cell Cycle Time (hr)	Reference
Basal	mouse	♂	8	F.L.M.	380	Blenkinsopp, 1969
Superficial	mouse	♂	8	F.L.M.	1400	Blenkinsopp, 1969
Basal	hamster	♂	adult	?	28	Boren & Paradise, 1976
Mucous	hamster	♂	adult	?	25	Boren & Paradise, 1976
Basal	hamster	♂	13	G.C.	159 ± 11	Boren & Paradise, 1978
Mucous	hamster	♂	13	G.C.	97 ± 3	Boren & Paradise, 1978

F.L.M. = Fraction Labelled Mitosis

G.C. = Grain Counting

TABLE 1.5

RESPIRATORY EPITHELIUM: CELL CYCLE PHASE TIMES

Airway Level - Cell Type	Animal	Sex	Age (wks)	Cell Cycle Phase Time (hr) t_S	t_{G2}	t_M	t_{G1}	Reference
Trachea	rat	♂♀	adult	8	-	-	-	Shorter et al., 1966
	rat: C.D.*	♂	5	7	3.5	-	-	Wells, 1970
- Basal	mouse	♂	8	8	3.6	-	-	Blenkinsopp, 1969
- Superficial	mouse		8	8	3.6	-	-	Blenkinsopp, 1969
- Basal	hamster	♂	adult	6-8	< 3	-	-	Harris, et al., 1975
- Mucous	hamster		adult	6-8	< 3	-	-	Harris, et al., 1975
- Basal	hamster	?	?	12	3.5	0.2-0.3	12	Boren & Paradise, 1976
- Mucous	hamster	?	?	7	2.9	0.2-0.3	15	Boren & Paradise, 1976
- Basal	hamster	♂	9-13	12	3.2	0.6	144	Boren & Paradise, 1978
- Mucous	hamster		9-13	10.5	2.5	0.6	87	Boren & Paradise, 1978

*C.D. = Conventionally Derived

Growth Fraction (GF) and resting cells (G_0): the growth fraction, that is the proliferating proportion of the cell population, can be determined with either the continuous labelling or double labelling techniques (see Section 4). The only such determinations for respiratory tract that have been reported are those by Boren and Paradise (1976, 1978). Their studies of hamster trachea showed that the GF consisted of 2 - 4% of basal cells and 1% of mucous cells. Studies in rat presented herein and by Donnelly et al., (1982), have shown that serous and indeterminate (ie. 'intermediate') cells also contribute to the GF in rat tracheal epithelium, but the extent of the contribution requires quantification.

Boren and Paradise (1978), observed among the proliferating basal and mucous cell populations, a very small proportion of cells that did not show grain halving with time after a pulse label with ^{3}H-T; this finding suggested the presence of small numbers of both basal (0.3%) and mucous (0.1%) cells which were either resting (ie. in the G_0 state) or at least proliferating more slowly than other labelled cells.

1.1.3. Differentiation

Epithelial cell renewal occurs as cells pass through proliferative, maturation and functional compartments during their lifespan. In 1881, Drasch suggested that dividing respiratory epithelial basal cells differentiated first into mucous then into ciliated cells without an intervening division. The contrary

suggestion that daughters of dividing basal cells matured first into ciliated columnar cells then into mucous cells, arose from observations of respiratory epithelia from asthmatic patients and experimental injury and repair in rabbit and rat trachea (Condon, 1942; Hilding, 1943).

The pathways of differentiation can be studied by a variety of cell kinetic techniques. Bindreitter et al. (1968), after using ^{3}H-T to pulse label a cohort of cells in the tracheal epithelium of young rats, observed changes in the pattern of labelling with time to study both proliferation and differentiation. The authors suggested that differentiation proceeded from basal to mucous and then to ciliated cells. A study of rat airway epithelium using the technique of continuous labelling (Section 1.4.3) led Blenkinsopp (1967), to suggest that basal cell division produced one basal and one superficial cell, ie. underwent asymmetric division where one daughter was committed to differentiation and the other remained in the basal compartment; it was further suggested that the superficial cell then produced two daughter cells, one of which was lost by sloughing.

After using several kinetic techniques (Section 1.4) to observe renewal in hamster tracheal epithelium, Boron and Paradise (1978), proposed a 3-compartment model: the first compartment of self-renewing basal cells, gives rise to a second consisting of mucous cells, some of which retain the ability to divide while others lose it and become fully differentiated. The third compartment consists of fully differentiated cells only, which do not divide, and have a finite life span.

A different system was proposed by Donnelly et al. (1982), who observed changes with time in numbers of labelled cells and combinations of labelled adjacent cell pairs, which, in theory, represent the recent progeny of a single cell's division. No significant changes occurred in labelling indices over a 10 day period. However changes in labelled pairs of adjacent cells suggested that: "a) ciliated cells can develop from cells of intermediate (indeterminate) morphology within 24 h; and b) that both superficial mucous (goblet) and ciliated cell development is preceded by 2 divisions, a basal cell division followed by an intermediate cell division". The authors suggested that the type of cell which results might depend on environmental influences.

The intermediate cell group deserves further comment: epithelial cells, which rest on the basement membrane with their apices in the lumen and have no distinguishing cytoplasmic features, were defined as intermediate cells (Jeffery and Reid, 1975); small numbers of these cells have been found to proliferate (Donnelly et al., 1982). It is important to note that McDowell et al., (1983), in a quantitative study of hamster trachea, showed that this cell group is difficult to classify accurately at the light microscope level and by electron microscopy was seen to comprise a heterogeneous population including secretory cells and 'tall' basal cells as well as rare true intermediate cells. This group has therefore been renamed indeterminate cells (McDowell et al., 1983; Jeffery and Corrin, 1984a).

The studies of McDowell and Trump (1983) have been concerned

with the histogenesis of preneoplastic and neoplastic lesions in tracheobronchial epithelium. From their observations of human tissue and parallel studies on normal epithelial kinetics and the reparative response following epithelial injury in animals, the authors conclude that secretory cell hyperplasia, stratification, epidermoid metaplasia, and 'carcinoma in situ' are closely related histogenetically. They suggest that the lesions arise from hyperplastic proliferation of mucus-secreting cells associated with phenotypic changes. Cell quantification and labelling experiments with ^{3}H-T indicated that 'pre-ciliated' cells were the progeny of secretory cell divisions and that many of the former still contained secretory granules carried over following division. The authors proposed that secretory cells play the major role in genesis of ciliated cells during fetal and neonatal development, during adult epithelial cell turnover and during epithelial regeneration.

1.1.4. Factors causing variation in proliferation and differentiation

Rates of proliferation, as measured by MI, REM, LI and TT, and pathways of differentiation have been found to vary with changes in both intrinsic animal characteristics and factors related to the external environment.

Intrinsic factors

Airway level: MI and LI decrease as the airways descend from the trachea (proximal) to the bronchiolus (distal), particularly in young and growing animals. In older animals the same trend is present but it is less pronounced (Bjork, 1960; Shorter et al.,

1964, 1966; Blenkinsopp, 1967; Divertie et al., 1968; Bolduc and Reid, 1976). TT, (as would be expected from the MI and LI data), increases with descending airway level (Blenkinsopp, 1967; Bolduc, 1976).

Animal strain: given the same sex, age (or weight) and airway level, MI, LI and TT vary between strains of mouse (Simnett and Heppleston, 1966) and rat (Blenkinsopp, 1967; Wells, 1970), although this difference may lessen with age (Simnett and Heppleston, 1966).

Sex differences: results show variation - Simmnet and Heppleston (1966), suggest that with increasing age there is a trend towards higher MI in females than in males in 3 mouse strains, although other studies of LI found no differences between males and females (Shorter et al., 1966; Divertie et al., 1968). In young (33 day old) rat airways, MI was greater and LI was doubled in males compared to females, but by 93 days these differences were absent. Interestingly the alveolar epithelial and migratory cells of the older rats again showed a higher MI for females than males (Bolduc and Reid, 1976).

The relationship of oestrogen levels to mucous cell numbers is of particular interest: variation with the oestrous cycle has been observed in rats (Hayashi, et al., 1981) and oestrogen administration can increase mucous cell numbers in guinea pig airways (El-Heneidy, 1976; El-Ghazzawi, 1979) suggesting that hormone levels may be important in either proliferation or differentiation in airway epithelium. These observations have lead McDowell and Trump, 1983, to the suggestion that the phenotypic

expression of lung carcinoma may be influenced by hormone levels.

Age: MI and LI tend to decrease and TT to increase, with age. Simnett and Heppleston (1966), observed 2 mouse strains, comparing young adults (3 month) with animals at the end of their natural lifespan (18 - 24 months of age): one strain (C57BL) showed a 60-90% reduction of their 3 month value; in strain A, males showed no significant difference whereas females showed an increase with age. The same workers found steadily decreasing LI with age, an observation confirmed by Evans et al., 1977. The studies of Bjork (1960), and Bolduc and Reid (1976), concentrated only on the early phases of growth (ie. to 3 months) where the decrease in MI coincided with the plateau of the growth curves for the animals. Blenkinsopp (1967), and Bolduc (1976) both demonstrated that in rats, TT increases with increasing age.

Growth factors: Data is now emerging about the role played in modulation of epithelial differentiation by the polypeptide TGF-β, originally purified from platelets and found to stimulate anchorage-independent growth of normal fibroblasts. This polypeptide, produced in a biologically inactive form by virtually all cell types, is active in many tissues; its structure and functions have recently been reviewed by Sporn et al., 1987. Studies of its effects on *in vitro* preparations of rabbit tracheal cells (Jetten et al., 1986) and normal human bronchial epithelial cells (Masui et al., 1986), show that TGF-β inhibits proliferation in a time and dose-dependent manner and causes cells to accumulate in the G_0/G_1 phase of the cell cycle. The biochemical markers used by these workers suggested that TGF-β induced terminal cell

division followed by squamous cell differentiation. Concurrent application of TGF-β and vitamin A resulted in decreased proliferation and lack of expression of the squamous differentiated phenotype. By contrast, addition of high levels of Ca^{++} stimulated squamous differentiation (Jetten et al., 1986). TGF-β applied to malignant bronchial cell lines did not decrease cell proliferation (Jetten et al., 1986; Masui, et al., 1986). The mechanism of action of this molecule is as yet unknown however both normal and malignant respiratory tract cells have high-affinity receptors for TGF-β (Masui et al., 1986). The observation that TGF- did not change intra-cellular levels of cyclic-AMP in those cells whose growth it effected, suggests a cyclic-AMP independent action on cell proliferation. Both groups of authors suggested that TGF-β is important in vivo, in the control of normal epithelial differentiation.

Cyclic adenosine-3,5'-monophosphate (cyclic-AMP) plays an important role in the balance between proliferation and differentiantion in many tissues. An increase in the intra-cellular concentration of cyclic-AMP is associated with increased cell proliferation in salivery gland epithelium, hepatocytes, Schwann cells, melanoma cells (Green, 1978) and decreased proliferation in dermal fibroblasts, adrenal cortical cells (Green, 1978), glioma cells (Sharma and Raj, 1987) and mammary carcinoma cells (Fontana et al., 1987). The production of cyclic-AMP is catalysed by a cell surface membrane-bound enzyme, adenyl cyclase (Rall and Sutherland, 1962), which is sensitive to growth factors and hormones (Birnbaumer, 1970). Cyclic-AMP, once released, mediates its intra-cellular actions via a cytoplasmic family of enzymes, the protein kinases (Hittleman and Butcher, 1971).

Because of the association between early cell proliferation and increases in intracellular concentrations of polyamines (marked by the activation of ornithine decarboxylase, ODC) and changes in cyclic-AMP levels in many tissues, Willey et al., 1985, studied changes induced in the levels of cyclic-AMP and the induction of ODC in normal human bronchial epithelial cells by **epidermal growth factor** (EGF), **pituitary extract** (PEX) and **epinephrine** (EPI). The results indicated that an increase in ODC activity was necessary but not sufficient and cyclic-AMP stimulation was unneccessary, ie. in that system, proliferation could occur by cyclic-AMP independent mechanisms.

Extrinsic factors

Diurnal variation in mitotic and labelling indices, well demonstrated in tissues such as epidermis and small bowel (Al-Dewachi et al., 1976; Clausen, 1979) has also been reported in the respiratory tract. In the latter a peak in either mitotic or labelling indices has been found in the morning in rats (Messier and Leblond, 1960; Romanova, 1966; Sokolova, 1975) and in the afternoon/evening in mice and hamster (Koburg, 1962; Kmoch et al., 1975; Das, 1979). Studies carried out under conditions of constant light by Boren (1970), and Boren and Paradise (1978), showed no significant diurnal variation in LI with time. Clearly, information available is limited and inconsistent. the source of variation, whether due to real differences or different methods of measurement, remains to be clarified.

Vitamin A status: Retinoids have been shown to play an important role in the control of epithelial proliferation and

differentiation including that of the respiratory tract. Wolbach and Howe (1925), demonstrated excessive cellular proliferation in respiratory epithelium of vitamin A-deficient animals which subsequently underwent squamous keratinizing metaplasia. Condon (1942), in a study of regeneration in normal and vitamin A-deficient rat tracheal epithelia, observed rapid epithelial proliferation: 8 hours after colchicine administration, 60% of epithelial cells were in mitosis compared with 'a few scattered mitoses' seen in normal epithelium. Harris et al., 1973, confirmed these observations, finding large increases in both MI and LI in vitamin A-deficient hamster trachea. In contrast, Sherman (1961), in a comparison of tracheal, corneal, and epidermal epithelia from vitamin A-deficient rats found a decreased MI in all the epithelia studied: MI returned to normal levels after local or oral application of vitamin A acetate. Lane and Gordon (1979), although observing foci of squamous metaplasia in vitamin A-deficient rat tracheal epithelium, reported no significant difference between the LI of rats on a normal diet and those deficient in the vitamin. Chopra (1983), examined the change in LI of basal and mucous cells in newly established tracheal explants from normal and deficient hamsters: mucous cells from deficient epithelia showed only an initial transient increase in LI, whereas basal cells maintained a high proliferative activity throughout the life of the explant and subsequently gave rise to squamous metaplastic cells.

An extensive study of vitamin A deficiency and restoration in hamster tracheal epithelium (McDowell et al., 1984a, b; Strum et al., 1985), showed the primary effect of deficiency to be a decrease in the proliferation rate of basal cells and (to a much

greater extent) mucous cells, with minimal morphological change; also described was the development of squamous lesions made up of epidermoid cells containing PAS positive granules, whose LI was raised above control levels. The authors concluded that: (i) vitamin A is necessary for maintenance of normal rates of cell proliferation and that mucous cells are particularly responsive to its absence. (ii) Prolonged vitamin A deficiency caused cell death which resulted in a reparative response. (iii) Mucous cells, which actively proliferate during repair, are capable of expressing keratin-manufacturing or mucous-secreting phenotypes, but vitamin A deficiency results in a predominance of the former, ie. epidermoid lesions rather than a muco-ciliary epithelium are produced.

In a study of the effect of restoration of vitamin A over 7 days, the same authors (McDowell et al., 1984 b) found that basal cell proliferation remained consistently less than controls, but mucous cell proliferation began to rise 2 days after restoration, as did the numbers of preciliated cells 24 hours later. Within 7 days, a restored pseudostratified epithelium with normal proportions of basal, mucous and ciliated cells was present. The results led the authors to suggest that the mucous cell plays a primary role in normal development and repair in tracheal bronchial epithelium.

Several authors have now studied disaggregated tracheal epithelial cells of rabbit (Jetten and Smits, 1985; Jetten et al., 1987) and hamster (Niles et al., 1986; McDowell et al., 1987a, b) in vitro, observing the differences between growth with and without vitamin A. All of these authors observed that cultures grown on a

thick gel base of type I collagen without vitamin A expressed a stratified squamous phenotype which bound biochemical markers of squamous differentiation. Cultures grown on collagen with vitamin A, had higher levels of proliferation than those without and differentiation of a muco-ciliary phenotype was promoted (Wu et al., 1985; Wu and Whitticot, 1986; Jetten et al., 1987; McDowell et al., 1987a).

The mechanism of action of vitamin A is still unknown. One suggestion has been that it increases the binding of epidermal growth factor (EGF) to cells through increases in the number of EGF receptor sites (Jetten, 1984). Alternatively, vitamin A may control gene expression by interaction with protein kinases, both the cyclic-AMP dependent and cylic-AMP independent varieties (Sporn et al., 1987).

1.1.5. Summary

1. In airway epithelium, basal, secretory (serous and mucous) and indeterminate cells comprise the proliferating fraction, although normally very few cells are dividing at any airway level, resulting in a long-lived epithelial surface (ie. with a slow turnover time).

2. The number of dividing cells varies, decreasing from proximal to distal airway levels and with increasing age. There are also differences between species and strains, and some evidence for inconsistent differences between male and female.

3. There is a diurnal variation in the number of proliferating cells.

4. Evidence suggests that endogenously produced growth factors such as TGF-β may be important in controlling the balance between proliferation and differentiation in normal airway epithelium.

5. Vitamin A is an important influencing dietary factor which specifically targets mucous cells, but the mechanism of its action is not yet understood.

6. There is very little detailed information about cell kinetics in airway epithelium.

7. Several studies have shown that differentiation pathways are as follows: from basal to mucous and ciliated cells and from mucous to ciliated cells; the latter are considered to be highly functional end stage cells.

8. 'Compartments' serving different kinetic roles have been suggested but their exact components and quantitative relationships and factors influencing their functional interactions are unknown.

9. Available data about airway epithelial renewal are mostly from animal studies and virtually nothing is known about human lung and its intrinsic variation and disease.

The present studies concentrate on detailed quantitation of normal proliferation and pathways of differentiation, and the changes induced in these parameters in a rat model of bronchitis induced by Tobacco Smoke.

1.2. TOBACCO SMOKE INDUCED CHANGES IN EPITHELIAL RENEWAL

1.2.1. Introduction

Numerous extrinsic agents, shown to affect cell division and thought to alter patterns of differentiation in airway epithelium, have been implicated in the aetiology of respiratory disease. Important information about mechanisms of epithelial renewal in response to injury can be gained from the use of these agents in experimental models. Foremost are the self-administered and airborne irritants such as tobacco smoke and the industrial and domestic environmental pollutants (eg. oxides of sulphur, nitrogen, ozone, and vehicle exhaust fumes); two other groups are chemical carcinogens and infectious agents. Observation of repair after mechanical trauma, and adminstration of certain therapeutic drugs which change proliferative activity provide further insights.

The nervous system and lymphocytes present in epithelium and bronchus-associated lymphoid tissue may also have a part to play in controlling cell division and their roles should be investigated further. Available information about the effects of these substances and procedures on proliferation and differentiation in the respiratory tract have been reviewed (Ayers and Jeffery, 1988), but as yet the lung remains relatively unexplored with regard to the factors which control proliferation and differentiation of its many distinct cell types both in health and disease.

In the following section, the effects of whole TS will be

examined in detail along with changes induced by exposure to some individual TS components.

1.2.2. Whole Tobacco Smoke

Tobacco smoke is a complex organic mixture which has been shown to damage each airway level in distinct ways, and comprises more than 2000 known compounds (U.S. Surgeon-Generals Report, 1979). Intensive investigation spanning two decades has yielded a large amount of data about the morphological and functional changes induced by exposure to whole TS in both experimental animal models and in human smokers. Both particulate and gaseous phases of TS contribute (Armitage and Turner, 1970; Robinson and Forges, 1975; Coggins et al., 1980; Betts et al., 1981).

Whole TS, when given daily to experimental animals for short periods of up to 12 weeks, causes an increase in epithelial thickness, mucous (goblet) cell hyperplasia in proximal airways (Lamb and Reid, 1969; Mawdesley-Thomas et al., 1971; Jones et al., 1972; Walker et al., 1978a; Hayashi et al., 1978; Betts et al., 1981; Jeffery and Reid, 1981) and an increase in size and number of alveolar macrophages (Walker et al., 1978b). Because secretory cells with transitional morphological features were found, Jeffery and Reid (1981), suggested that the increase in mucous cell number was largely due to transformation of existing serous cells, ie. the cytoplasmic enzymes concerned with mucin synthesis were modified. However, to understand what proportion of the response is due to transformation and proliferation respectively, a quantitative autoradiographic analysis is necessary. Continued exposure may

lead to loss both of cilia and goblet cells, basal cell proliferation, and focal squamous cell metaplasia which, with full thickness cellular atypia, is regarded as 'carcinoma in situ' (Leuchtenberger and Leuchtenberger, 1960; Auerbach et al., 1961, 1970, Hammond, 1970; Harris and Negroni, 1967; Dontenwill et al., 1973; Kobayashi et al., 1974; Davis et al., 1975 a, b; Reznik-Schuller et al., 1975; Bernfield et al., 1979; Dalby et al., 1980). Similar findings have been described in man (Auerbach et al., 1979).

Despite the volume of this data, quantitative information about TS-induced changes in the proliferation and differentiation processes of airway epithelium, is still sparse.

The effects of TS on MI and LI have been studied only after short-term exposure. In all species studied, MI increases in response to TS in both extrapulmonary and intrapulmonary airways, but to a greater extent in the former (Jones et al., 1972; Bolduc and Reid, 1976; Bolduc et al., 1981). Nearly all studies have utilized male animals and although differences have been reported in the mucous cell hyperplastic response of male and female rats to TS (Hayashi et al. 1978, Huber et al., 1981), no similar comparison has been made for MI or LI. Both Lamb and Reid (1969) and Wells and Lamerton (1975) found a dose-related response to TS, the highest number of cigarettes smoked producing the greatest number of mitotic divisions. The peak increase in mitotic index was at 24 hours, which in spite of continued exposure returned to control levels by about 2 days (Wells and Lamerton, 1975). If, however, there was a break in TS exposure, a second peak was generated on

recommencement (Jeffery and Reid, 1980; Bolduc et al., 1981).

The effect of TS on LI has been studied in hamsters by Boren (1971) who found a significant increase in LI (up to 4 fold) in airways of 400 μm or less, after only 8 hours of TS. A second peak in LI was found at 40 hours of exposure, in the alveolus but not in the conducting airways; trachea and main bronchi were not examined in the study.

There are no published studies of TS-induced changes in length of each cell cycle phase.

1.2.3. TS Components

Despite the small amount of data about TS-induced changes in cell renewal in airway epithelium, there are numerous studies on cell kinetic changes due to single components of TS such as nitrogen oxides and various chemical carcinogens eg. benz-(α)-pyrene and the nitrosamines.

1.2.3.1. Nitrogen dioxide (NO_2): extensive studies by Evans and his colleagues have shown that the primary site of damage by NO_2 is the terminal and respiratory bronchioli and adjacent alveoli (Cabral-Anderson et al., 1977). All studies showed that ciliated cells of the terminal bronchioli and type I cells of proximal alveoli are destroyed (Stephens, 1974a, b). Subsequently, nonciliated bronchiolar (Clara) cells and alveolar type II cells increased their rate of proliferation within 24 hours (Stephens,

1974a; Hackett, 1979), returning to control levels by 2-4 days (Evans, 1972; Stephens, 1974a). The proliferating Clara and type II cells differentiated into ciliated and type I cells respectively (Evans et al., 1975). Thus 'short term' exposure (eg. daily for 40 days) induces bronchial and bronchiolar epithelial hyperplasia with tall columnar epithelium and frequent mitotic figures; longer (ie. lifespan) exposure induces emphysema (Freeman et al., 1968).

A recent study (Evans et al., 1986) concentrated on the effects of NO_2 on proximal intrapulmonary bronchi. One hour after injection of ^3H-T, basal and nonciliated columnar cells ie. serous, indeterminate and mucous cells, were labelled. All of these cells except basal cells, were stimulated to divide by NO_2. After cell division, cells of all types were labelled. It was concluded that the primary progenitor cell of the large airway was secretory, not basal.

1.2.3.2. Chemical carcinogens: of the polycyclic aromatic hydrocarbons in TS, benz-(α)-pyrene (BP) and some nitrosamines have been widely studied, although few papers have examined the early proliferative changes induced in the respiratory tract. Binding of tritiated BP occurs with DNA of ciliated, mucous and basal cells, and is dependent on enzyme activation (Harris et al., 1973; Stoner et al., 1978). During vitamin A deficiency, which has been linked to increased risk of developing carcinoma (Basu et al., 1976), binding is increased and appears to be greatest in areas of squamous metaplasia (Harris et al., unpublished observation quoted in Genta et al., 1973).

In an in vitro study using neonatal rat tracheas, Crocker et al. (1965), found that exposure to BP, and other carcinogens at a range of doses and for a maximum of 14 days, caused loss of differentiated (superficial) cells and early increases in both MI and LI in the remaining basal cells. Continuation of exposure with the higher doses resulted in the production of squamous metaplastic changes. It was found that intratracheal administration of BP, repeated weekly for up to 20 weeks, gave rise to early cell proliferation of either basal (Reznik-Schuller, 1974 a, b) or mucous cells (Becci et al., 1978b). In either case these changes preceded the development of squamous metaplasia, keratin formation and premalignant changes.

The action of nitrosamines, in particular diethyl nitrosamine, (DEN) on airway epithelium has been studied in some detail (Reznik-Schuller and Reznik, 1980; Reznik-Schuller, 1976a, b; 1977a, b). In hamster trachea, DEN induced basal cell proliferation, mucous cell differentiation and epithelial cell hyperplasia, followed by development of papillary polyps made up of mucous and 'intermediate' cells. In hamster bronchi, several nitrosamines were found to specifically affect Clara and APUD cells, inducing their proliferation and the subsequent production of squamous metaplasia (Reznik-Schuller and Reznik, 1980).

1.2.4. Summary

1. Despite intensive study of the morphological effects of short and long term TS exposure, there is little information on

changes induced by TS in airway epithelial cell proliferation and differentiation.

2. TS exposure causes an increase in MI peaking at 24 h, returning to normal by 48 h. The response is dose-dependent and occurs with varying intensity, in both extra and intrapulmonary airways.

3. LI increases in alveoli during TS exposure.

4. The TS components NO_2, benz-pyrene and nitrosamines have been shown to stimulate proliferation of basal, mucous, Clara and type II cells.

5. There is no information about changes in the cell cycle and phase times or about factors controlling the proliferative response.

1.3. INFLUENCE OF DRUGS ON TS-INDUCED PROLIFERATION AND DIFFERENTIATION

While drugs such as isoprenaline sulphate and pilocarpine have been shown to stimulate airway epithelial cell division (Bolduc and Reid, 1978; Jones and Reid, 1979) others inhibit it and therefore may be of use in inhibiting TS-induced changes in cell proliferation. Of these, several nonsteroidal and steroidal drugs have been shown to inhibit the increase in mucous cell numbers due to irritation by tobacco smoke (Jeffery et al., 1982; Rogers and Jeffery, 1986, a, b). Although the mechanism of this response is

not yet elucidated, these drugs could produce their effect either by increasing secretion or decreasing synthesis of mucus, or by inhibition of proliferation of mucous cells. Supportive evidence for the latter mechanism comes from work using other organ systems which shows that the nonsteroidal anti-inflammatory drug Indomethacin inhibits increases in the LI of rat epidermis induced by promoting agents such as 12-O-tetradeca-inoylphorbal-13-acetate (TPA); LI increases in kaolin-induced granulomata and methyl cholanthrene-induced tumours were also found to be inhibited (Francis and Marks, 1977; Furstenberger and Marks, 1978). Interestingly the proliferative response to TPA is restored by addition of PGE_2 but not PGF_2 (Francis & Marks 1977; Furstenberger & Marks, 1978; Furstenberger et al., 1979; Fulton & Levy, 1980).

In the respiratory tract the anti-tussive agent Phenylmethyloxadiazole present as 2% by weight of tobacco partially inhibited increases in MI seen at 2 weeks of exposure to tobacco smoke (Bolduc et al., 1972; Jeffery and Reid, 1981). Paradoxically at 1 week the increase was delayed but greater than that seen with tobacco smoke alone (Bolduc et al., 1981).

Apart from the inhibitory effect of Dexamethazone on LI of fetal (16-18 day) mouse lung tissue (Kauffman et al., 1977b), there is little known of the effects of steroids on pulmonary cell division. Early administration of Methyl prednisolone to adult mice inhibited butylated hydroxytoluene-induced type II cell proliferation (as measured by LI) providing the degree of lung injury was 'mild' (Smith & Brody, 1981).

Colchicine and the Vinca alkaloids, Vinblastine and Vincristine, are classic inhibitors of cell division arresting mitosis at metaphase (Eigsti and Dustin, 1955; Clark, 1971). In this context it is of interest that vinblastine sulphate (given sub-cutaneously at 0.05 mg/Kg daily for 21 days) prevented tobacco smoke-induced mucous cell proliferation in the upper trachea and proximal airways of SPF rat lung. However, at the dose used, vinblastine did not appear to be inhibiting cell division as there was no accumulation of metaphase arrests with time (Evans et al., unpublished; Jeffery et al., 1984), suggesting an alternative mechanism of action such as inhibition of mucous synthesis (Adler et al., 1984).

Some recent findings regarding the drug N-acetylcysteine (Nac) are of interest. This substance was first found to be effective as an antidote for Paracetamol poisoning (Prescott, 1979; Ho and Beilin, 1983) and subsequently used as an oral and parenteral mucolytic agent due to its ability to decrease mucous viscosity in vitro (Sheffner, 1963; Hurst et al., 1967; Hirsch et al., 1969; Lightowler and Lightowler, 1971; Marriott and Richards, 1974) and in clinical studies with patients (Hirsch and Kory, 1967; Hurst et al., 1967; Hirsch et al., 1970; Pulle et al., 1970). It has been suggested that the mucolytic ability is due to the breaking of disulphide bonds which create the complex structure of glycoproteins (Havez et al., 1970).

Recent studies suggest that the uses of this agent are diversifying. It is a free radical scavenger (Bernard et al., 1984)

and it increases the intracellular stores of glutathione. Being a powerful intracellular reducing agent present in many tissues, it protects cells from toxic damage by combining preferentially with electrophilic metabolites before irreversible alkylation occurs (Prescott, 1982). It is possible that the protective role played by Nac in several experimental models is mediated through these mechanisms: liver and heart cells were protected from acetaminophen and adriamycin toxicity as were liver cells from paracetamol damage (Mitchell et al., 1973; Olson et al., 1980; Prescott, 1982); it prevented TS-induced decrease in phagocytosis by alveolar macrophages (Green et al., 1968) and was thought to both protect an in vitro preparation of L2 lung epithelial cells from polymorphonuclear leukocyte mediated oxidant injury and decrease the susceptibility of these cells to the damage (Simon and Suttorp, 1985). In addition it has been shown that Nac decreased TS-induced secretory cell hyperplasia (Rogers and Jeffery, 1986a). The question of whether glutathione plays a role in control of cell proliferation is as yet unanswered (Higashi and Yamaguchi, 1983), but the latter observation suggests that Nac could act via a direct or indirect effect on this mechanism. It was therefore decided to test whether Nac could modify the TS-induced changes in airway epithelial proliferation, and a description of these experiments is presented in Chapter 5.

1.4. TECHNIQUES FOR ASSESSMENT OF CELL RENEWAL

The use of simple methods to measure proliferation based on quantitation of dividing cells has been discussed briefly in Section 1.1.2.1. The theoretical possibilities of some more complex

means of measuring kinetic parameters in renewing cell populations will now be discussed. Extensive discussion of standardizing procedures, the problems and some solutions, involved in the use of these techniques, is available in Wright and Alison, (1984), in the section on methodology.

1.4.1. Fraction of Labelled Mitoses (FLM)

This method was developed by Quastler and Sherman in 1959, and enables cell cycle time (T_C) and the duration of each cycle phase (t_{G1}, t_S, t_{G2}, t_M) to be measured. Proliferating cells in a population are labelled by a single pulse of ^{3}H-T and followed with time as they move through G_2 into mitosis to produce a cohort of labelled mitotic figures. Consecutive samples at short time intervals after the pulse, and subsequent autoradiographic analysis of the population, allows the rise and fall in the percentage of labelled mitotic figures with time to be plotted (ie. the FLM curve). If all cells in the population are cycling asynchronously and at the same rate, a well-defined curve is generated rising from 0 to 100% and back to 0% of labelled mitotic figures with each complete passage of the originally-labelled cells around the cycle. The length of each cycle phase and T_c can be calculated from measurements made of the FLM curve (Quastler, 1962; Barret, 1970; Mendolsohn, 1971; Gilbert, 1972; Steel, 1972; Shackney, 1974; Hartman, 1975; Aherne et al., 1977).

1.4.2. Double Labelling

This technique enables t_S and the rate of entry into DNA

synthesis to be calculated by using both ^{3}H- and ^{14}C-labelled-thymidine. The energies, and thus 'path lengths' of each isotope are sufficiently different to enable cells labelled with either isotope to be distinguished by autoradiography (Wimber and Quastler, 1963; Shultze et al., 1976; Aherne et al., 1977). A cell cohort is given a single pulse of ^{3}H-T (short path length) and after a known time interval (t) which must be shorter than t_S, a single pulse of ^{14}C-T (longer path length). The distinctly labelled cell populations can be subsequently differentiated and counted by having two superimposed layers of autoradiographic emulsion, one of which is too distant to be affected by ^{3}H-T. Assuming that the rates of entering and leaving the S phase are equal (ie. the cell population is in a steady state), t_S can be calculated (Aherne et al., 1977).

1.4.3. Continuous Labelling

This procedure enables the growth fraction, t_S, the rate of entry into DNA synthesis and tc to be calculated (Gereke, 1976; Aherne et al., 1977). A cell population *in vivo* or *in vitro* is 'continuously' exposed to a low level of ^{3}H-thymidine such that each cell in the growth fraction, as it comes into the S phase, becomes labelled. This can be done *in vivo* either by continuous infusion (Mendelsohn, 1962; Gereke, 1976) or by injections of low-dose ^{3}H-T at frequent intervals each shorter than t_S (Aherne et al., 1977). If the proportion of labelled cells is plotted against time, the curve first rises and then reaches a plateau when all of the growth fraction is labelled. t_S can then be calculated from the

slope of the curve, which measures the rate of entry into the S phase and thus allows use of the equation:- $t_S = LI/r_S$. The time at which the plateau occurs is equivalent to $(t_{G2} + t_M + t_{G1})$. If this summed time as read from the graph is added to the calculated t_S, the cell cycle time can be derived.

1.4.4. Grain Counting

For this technique, the mean number of silver grains over individual cell nuclei resulting from a single pulse label of ^3H-T, is plotted against time. The halving that results from a mitotic division occurs after a time equivalent to $t_S + t_{G2} + t_M$. If the plot of mean grain number against time is extended, the next halving takes place after a time equivalent to T_C (Koburg, 1963; Steel,1963; Freid, 1968).

1.4.5. Recent Developments

Recent years have seen the the development of new techniques to measure DNA content and cell cycle phase duration. Certain stains react stoichiometrically with DNA and in the past the Feulgen stain has been used extensively to measure the amount of light absorbed by each stained nucleus, at a specific wavelength, using an integrating microdensitometer. This method is laborious and the results difficult to analyse. It has in large part been superceded by the use of flow cytometric methods and the Fluorescence Activated Cell Sorter (FACS). This instrument enables individual (ie. disaggregated) cells, stained with a fluorescent

dye (eg. ethidium bromide or propidium iodide) to be passed in suspension through and excited by a laser beam. The fluorescence produced is detected and the resulting data expressed as a distribution of DNA content. This method has the advantages of being quick and precise. The amount of S-phase DNA may also be determined by incorporation of a thymidine analogue, bromodeoxyuridine (BUdR), and its detection in tissues with fluorescein-labelled monoclonal antibodies to BUdR, using either immunofluorescent techniques or quantitative FACS analysis (Gratzner et al., 1982, Dolbeare et al., 1983). The method can be applied to cell suspensions grown *in vitro* or to suspensions derived from sections of paraffin-embedded material (Hedley et al., 1983). The limitations of the method have been discussed by Vindelov et al. (1983).

1.5. SPECIFIC AIMS AND OBJECTIVES OF THE PRESENT STUDY

The aim of the present study was to examine experimentally mucous cell hyperplasia due to sub-acute tobacco smoke exposure focusing on the distinct processes of cell proliferation and differentiation and to examine the modifying effect, if any, on these responses by the drug N-acetylcysteine. The variable used to measure proliferative activity in tissue sections was labelling index following administration of tritiated thymidine to the animal. Relatively sophisticated techniques involving use of a flourescence activated cell sorter were not available for use at the time the study was initiated.

The specific objectives were to:-

1. induce mucous cell hyperplasia in extrapulmonary airways of the rat by exposure to an atmosphere of tobacco smoke;

2. measure the overall and relative proliferative response of all epithelial cells and the relative responses of morphologically distinct cell types by giving ^{3}H-thymidine to animals 1 hour before death to label the S phase of cycling cells;

3. assess pathways of differentiation by giving a single dose of ^{3}H-thymidine to control and tobacco smoke-exposed animals at the beginning of the experiment only and subsequently comparing changes in the distribution and partition of label between epithelial cells with time;

4. introduce N-acetylcysteine into the system in a prophylactic regimen and quantitatively assess its modification of the TS-induced response.

1.6. SUMMARY

1. The various cell types present in airway epithelium and their proliferative potential have been described.

2. There is relatively little proliferative activity in normal healthy lung but there is ample capacity to recruit rapidly non-cycling cells into the cell cycle.

3. Factors contributing to the variation seen in base-line proliferation rats include: species, sex, hormonal status, airway level, age, diurnal rhythm and pathogen-free status.

4. Under some circumstances (eg. Vitamin A deficiency and NO_2 injury) secretory cells (mucous and Clara) are proliferative and their role in epithelial repair has previously been underestimated.

5. A variety of stimuli increase proliferation as part of a reparative process, eg. oxidants, chemical carcinogens or certain drugs, some of which stimulate division whilst others inhibit the proliferative response to irritation.

6. An increase in the number of some cell types may appear due to 'differentiation' rather than proliferation and a 3-compartment model can be applied to airway epithelium comprising: (i) the self renewing/proliferating compartment made up of basal and secretory cells in the large airways, nonciliated bronchiolar (Clara) cells in the small airways, and type II cells of the alveolus; (ii) the differentiation compartment containing mature functional cells some of which retain the capacity to divide: eg. serous, mucous (goblet), Clara, type II cells; (iii) the fully-mature end stage cells which do not divide, eg. ciliated cells.

7. The present experimental study focuses on mucous cell hyperplasia due to sub-acute exposure to TS, examines the

contribution of proliferation and differentiation in the response in a number of cell types and determines the modifying effect of the drug N-acetylcysteine.

CHAPTER 2 – MATERIALS AND METHODS

2.1. ANIMALS AND HOLDING CONDITIONS

All animals used in these experiments were outbred male specific pathogen free (SPF) Sprague-Dawley rats, weighing between 180-220 g, obtained from OLAC, U.K. and Charles River, U.K. The animals were Grade IV (Medical Research Council, 1974) SPF, caesarian-born and barrier maintained. The importance of using SPF animals lies in the susceptibility of conventionally derived rats to chronic respiratory disease, which alters the proportions of airway epithelial cells, in particular increasing the numbers and changing the distribution of mucus-secreting cells (Reid, 1970; Bolduc and Reid, 1976).

After arrival in the animal house, the animals were allowed to settle for 24 hours before being weighed on an electronic balance (Mettler PK 480 Range, Gallenkamp and Co. Ltd.), marked and randomly allocated to experimental groups (Tables 2.1, 2.2, & 2.3). Each experimental regimen was initiated after an interval of 3 to 10 days. The animals were weighed each day during the experimental period, immediately prior to TS exposure. They remained in good condition and gained weight during the experimental procedures (see Chapters 3.2, 4.2 and 5.2).

The animals were kept isolated from all other animals in a positive pressure ventilated room and to prevent cross-infection from animals located elsewhere in the animal house, their cages were cleaned regularly by the experimenter only. They were either housed in reinforced polythene cages with 'low-dust' wood chip bedding (Experiments 1-4), or held in wire mesh cages, the base of which was

raised above sterilized bedding. The wire mesh cages were mounted in laminar flow cabinets (Forth Tech Services Ltd., Scotland) which continuously filtered and recirculated the room air. The animals received Lab. Chow 41B (Heygate and Sons, U.K.) and water ad libitum, except during the experimental procedures, when food and water were removed from both control and treated groups. They were maintained on a 12 hour light/12 hour darkness cycle in sequence with the outside day/night cycle.

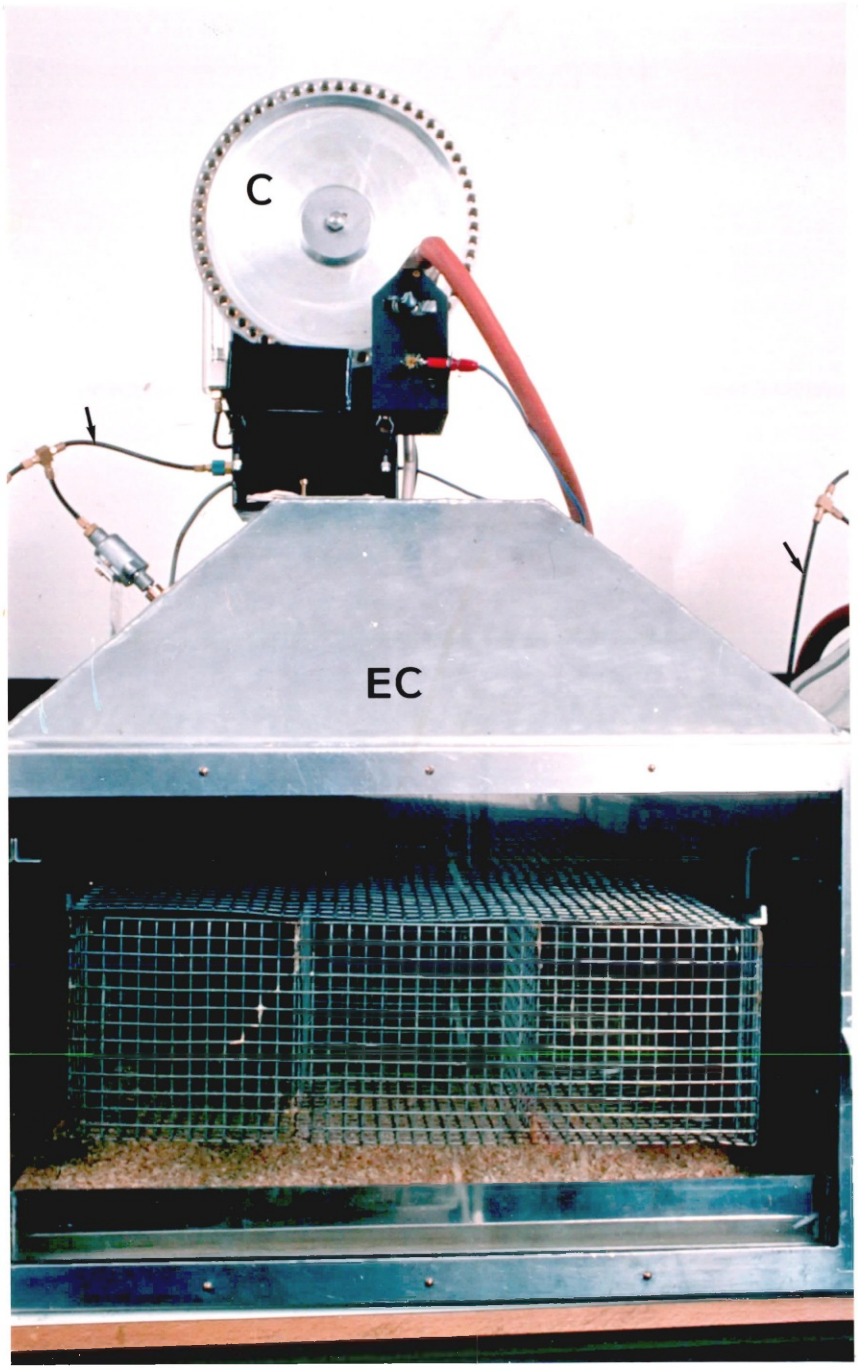
2.2. EXPERIMENTAL PROCEDURES

2.2.1 Exposure to Tobacco Smoke (TS)

During the daily exposure of the TS-treated animals, control animals remained in their cages without food or water, in the same room as the smoking machines, which exhausted all smoke from the exposure boxes to the outside of the room. During Experiment 5, filter-containing laminar flow cabinets were installed in the smoking room and control animals were held therein. Sham-exposure was not carried out, as previous studies had shown no significant differences in secretory cell number between sham-exposed animals and control animals kept in their cages (Jones et al., 1972).

The animals exposed to TS were treated for 4 hours daily, for 14 consecutive days with the smoke of 25 cigarettes delivered into aluminium exposure cabinets. The cabinets held a maximum of 18 animals and the TS was delivered by a modified Wright auto-smoking machine (Fig 2.1) (Wright, 1972). Two sets of replicate cabinets

Figure 2.1 Modified Wright Auto-Smoking Machine showing aluminium exposure cabinet (EC), catherine wheel for cigarettes (C) and pump lines (arrows).


C
EC

and machines were used: one at The Cardiothoracic Institute, Fulham Rd., London, SW3, for Experiments 1&3 (Tables 2.1 and 2.2); the other, constructed by the Engineering Department, Chelsea College, Manresa Rd., London, SW3, was used for Experiments 2&4 and Experiment 5 (Tables 2.1-2.3). The machines were operated by pump-generated air pressure (Charles Austen Pumps Ltd.) and adjusted with a timing box so that the burning time for 1 complete cigarette was 6 minutes; this was followed by the delivery of room air for 4 minutes before the next cigarette was automatically lit. Smoke was delivered as a 30 ml puff over 2 seconds produced by a 20 l/min jet of air passing through a small bore tube at right angles to the cigarette butt; this occurred 4 times per minute. An additional 5 l/min of room air constantly flowed through the cabinets which were continuously exhausted from 2 side ports. During each exposure, the humidity and temperature were monitored inside the cabinets (the former with a hair hygrometer, Gallenkamp and Co. Ltd., and the latter with a maximum-minimum thermometer) and showed a range of 40-80% and 21-27 ^{o}C, between the beginning and end of the TS exposures. During the time over which these experiments were conducted, two brands of cigarette were used. For all except one study, 'John Players Navy Cut Plain' (ie. unfiltered) with a reported tar rating of 18-25 mg/cigarette, a nicotine yield of 1.4-1.9 mg/cigarette and a CO content of 12.5 mg/cigarette (Laboratory of the Government Chemist, 1978-1981) was the cigarette of choice. Capstan Full Strength cigarettes, tar, 26 mg/cigarette, nicotine 2.7 mg and CO 16.7 mg (Laboratory of the Government Chemist, 1982), were used in Experiment 5 when the tar and nicotine levels of the 'John Players' were found to have decreased.

During Experiment 5, CO and particle density levels and plasma CoHb concentrations were measured. Using a portable analyser, an Ecolyser 4000 (Energetics Sciences, Inc. N.Y.), carbon monoxide levels in the cabinets were observed to rise from 7 ppm to 120 ppm over the four hour exposure period. The CO concentration in the room was found to be 7 ppm. Particle density in both room and cabinets was measured concurrently with CO levels, using a Reading Dust Monitor (Holt and Young, 1964); this machine uses a collimated beam of light which is scattered by sample particles and the degree of scatter, and hence particle concentration, is determined by photomultiplier tubes. The output current of the tubes is displayed by an ammeter from which readings were noted and expressed in 'arbitrary units'. The particle level in the cabinets rose in tandem with the CO concentration as cigarettes were smoked.

After obtaining blood by cardiac puncture, plasma COHb levels were measured from 6 rats using an IL 182 CO-oximeter (Instrumentation Laboratory, USA) on the last day of a 14 day exposure. They were found to be 11.7 (SEM $\pm$1.1%) after 1 h and 16.4 (SEM $\pm$0.4%) after 4h of TS exposure (p 0.5) (Rogers, 1984; Rogers and Jeffery, 1986a). In similar experiments, the blood nicotine levels were measured and found to be 0.3 (SEM $\pm$0.3) ng/ml in control animals and 102.5 (SEM $\pm$32.1) (range 62-213) ng/ml ($p < 0.001$) in animals exposed to TS for 5 days (Rogers, 1984; Rogers and Jeffery, 1986a). The positions of the animals were changed daily, both within their cages and between cabinets, to compensate for varying humidity and temperature as well as possible non-uniform distribution of smoke within and between cabinets.

2.2.2. Dose of Tritiated Thymidine (^{3}H-T)

A preliminary experiment was carried out to establish the minimum dose of tritiated thymidine (^{3}H-T) (given as an aqueous sterile solution of methyl-^{3}H-thymidine of high specific activity: 25 Ci/mmol (ie. 925 GBq/mmol, Amersham Radiochemical Centre, U.K.) which would give adequate cell labelling in the autoradiographs of 1 μm thick plastic-embedded sections, yet inflict minimum damage upon dividing cells.

A total of 7 male SPF Sprague-Dawley rats were used: ^{3}H-T, at doses of 0.5, 1.0, 2.0, or 3.0 Ci/g body weight was given, i.p., (n = 2, 2, 1 and 2 animals respectively). The dose levels were based on those used in studies of gastro-intestinal epithelium (Cheng and LeBlond, 1974, I-IV). One hour later the rats were killed and the trachea, left lung and 1 cm of ileum (used to check cell labelling in the rapidly dividing crypt epithelium) were removed and processed for electron microscopy (see Appendix 3). From each animal, 1 μm sections were cut from randomly selected blocks of ileum and main bronchus as this airway level was used for all experimental procedures reported herein. Both were mounted side by side on the same slide and stained with Toluidine Blue (Section 2.3). Autoradiographs (ARs) were prepared and developed (Section 2.4.1) after incubation in the dark at 4^{o}C for 1, 3, 7, 10, 14 and 21 days. After qualitative consideration of the number of grains over cell nuclei, incubation time and background labelling over the sections of main bronchus and ileum from each animal, it was decided that a ^{3}H-T dose of 1 μCi/g, combined with an incubation

time of 14 days were the conditions that gave satisfactory labelling with a minimal background of AR grain production.

2.2.3. Studies of Proliferation and Pathways of Differentiation

Chapters 3 and 4 report results from 4 experiments, the plans of which are shown in Tables 2.1. and 2.2. Experiments 1&3 were conducted concurrently, as were Experiments 2&4 at a later date. Each experimental set comprised time studies aimed at examination of changes in proliferation and differentiation. Experiments 1&3 were pilot experiments involving small animal numbers. Available space in the smoking machines precluded the use of large numbers of animals in each experiment.

Changes in the types and proportions of proliferating airway epithelial cells with time were determined in both control - ie. unexposed animals killed at the beginning (day 0) and end (day 14) of the experiment - and TS-exposed animals, following the i.p. administration of a single pulse of ^{3}H-T (dose: 1 μCi/g), given 1 hour prior to death (Experiments 1 and 2, Table 2.1). The day 0 control animals (Experiment 2) were killed in 2 groups 12 h apart to observe possible diurnal variation. The TS-treated animals were killed after 1, 3, 7, 10 and 14 days of exposure.

Studies aimed at determining the pathways of differentiation in airway epithelium were undertaken in both unexposed and TS-exposed animals (Experiments 3 and 4, Table 2.2), by administration of a single pulse of ^{3}H-T (dose: 1 μCi/g) only, given at the beginning of the experiment (ie. on day 0). Animals

TABLE 2.1

PROLIFERATION EXPERIMENTS: NUMBERS OF ANIMALS

Time (days)	EXPERIMENT 1		EXPERIMENT 2		COMBINED NUMBERS	
	Control	Tobacco Smoke	Control	Tobacco Smoke	C	TS
0	-	-	7	-	7	-
1	-	-	-	4	-	4
3	-	2	-	1	-	3
7	-	2	-	1	-	3
10	-	-	-	5	-	5
14	-	2	3	2	3	4
		6	10	13	10	19

TABLE 2.2

DIFFERENTIATION EXPERIMENTS: NUMBERS OF ANIMALS

	EXPERIMENT 3		EXPERIMENT 4		COMBINED NUMBERS	
Time (days)	Control	Tobacco Smoke	Control	Tobacco Smoke	C	TS
0	-	-	7*	-	7*	-
1	-	-	3	3	3	3
3	2	2	3	3	5	5
7	-	2	3	5	3	7
10	-	-	3	5	3	5
14	2	2	3	5	5	7
	4	6	22	21	26	27

*From Experiment 2.

TABLE 2.3

EFFECT OF N-ACETYLCYSTEINE: NUMBERS OF ANIMALS

EXPERIMENT 5

PROLIFERATION

Time (days)	Control	NAC-Only	TS-Only	NAC & TC	Total Nos.
0	5	-	-	-	
1	-	3	3	3	
3	-	3	3	3	
7	-	3	3	3	
10	-	-	3	3	
14	5	4	4	4	
	10	13	16	16	55

DIFFERENTIATION

Time (days)	Control	NAC-Only	TS-Only	NAC & TS	Total Nos.
14	4	3	3	3	13
					68

from each group were subsequently killed at days 1, 3, 7, 10 and 14 without further administration of ^{3}H-T. Thus, a cohort of proliferating cells was labelled enabling changes in the distribution of label between cells to be followed by AR with time and pathways of differentiation traced. This technique and the limitations it imposes on interpretation is discussed in Chapter 7.

A total of 75 animals was used in the 4 experiments. The control values for proliferation and differentiation are identical and are represented by the data at the day 0 time point. They form a common baseline from which subsequent changes in both proliferating populations and pathways of differentiation with time can be assessed.

2.2.4. Effect of Nac

Chapter 5 presents the results of Experiment 5 (Table 2.3), conducted to test the effects of the mucolytic drug N-acetylcysteine (Nac) (Zambon, S.p.A, Milan) on TS-induced changes in airway epithelial cell proliferation and differentiation. A total of 68 animals was used, divided into 4 groups: Control, Nac-only, TS-only and Nac+TS. TS exposure was carried out as described in Section 2.2.1. Nac was given orally in the drinking water as a 1% solution (1g in 100 ml tap H_2O), the dose suggested by the manufacturers. The pH of the solution was adjusted to 6.5, equivalent to that of the tap water used to make up the solution. Free access to water containing Nac commenced 2 days before the 14 day TS exposure began, at which point the animals had a mean weight of 254 (SEM $\pm$5) g. The water and Nac solutions were changed daily

but for each weekend, when they were changed after 2 days. Rats, weighed daily, were housed 4 to a cage, each cage with one water bottle. Bottles were weighed daily before and after refilling, enabling the water intake of rats receiving Nac (with and without TS) to be monitored and the dose of Nac per animal to be calculated. This was done using the formula:

$$\text{dose Nac} = \frac{\text{mean ml } H_2O \text{ per rat} \times 10^4}{\text{mean rat weight}} \quad \text{mg/Kg body weight}$$

Water intake of the control and TS-only groups was not monitored directly but measurements in other concurrently running experiments (Rogers, 1984), showed that the mean daily intake per animal in these two groups was 34.0 ml (SEM $\pm$ 1.7) and 38.6 ml (SEM $\pm$ 2.1) respectively. For the animals given Nac, the daily water intake reduced at first then rose to a plateau between 1 and 4 days after the start of TS exposure. It reached a mean value of 24.7 ml (SEM $\pm$ 1.9) per animal per day for the Nac-only group and 21.5 ml (SEM $\pm$ 2.8) in the TS+Nac group. The calculated mean daily dose of Nac given as a 1% solution was 873.2 mg/kg (SEM $\pm$ 59.2) body weight (Table 2.4).

In each group of animals, the number of each cell type per 10 high power (100X oil immersion) fields was counted on days 0 and 14 only. Cells were identified by the morphological criteria described in Chapter 3, Section 3.3, as ciliated, serous, mucous, transitional (comprising two groups, sero-mucous and muco-serous), indeterminate and basal cells. The effects of Nac on TS-induced proliferation were assessed by quantitative autoradiographic analysis of tissue samples taken sequentially during the experiment

(days 0, 1, 3, 7, 10 and 14) from animals given ^{3}H-T 1h prior to death, as described in Sections 2.4 and 2.5. The modifying effects of Nac on TS-induced changes in differentiation were observed by comparing the cellular distribution of label produced by a single ip pulse of ^{3}H-T on day 0 in control epithelium, with the distribution observed 14 days later in animal groups exposed to Nac-only, TS-only or Nac+TS, without subsequent administration of ^{3}H-T.

2.3. HISTOLOGICAL PROCEDURES

All animals were killed 20 hours after the last exposure to TS by an overdose i.p. injection of Nembutal (Sodium pentabarbitone 60 mg/ml, May and Baker Ltd., London). The time interval allowed any depleted intracellular stores of glycoprotein to be reconstituted so that the secretory cells could be detected and distinguished by their differential uptake of Toluidine Blue stain; their numbers could thus be counted (Jeffery and Reid, 1981). The lungs were inflated with fixative (2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, EM Scope Ltd., London) *in situ* via the trachea until the pleural margins became sharp, thus approximating a uniform distention pressure. The trachea, main bronchus and lungs were removed *en bloc*. Tissue blocks were taken from the lower trachea, left exprapulmonary main bronchus, axial pathway and distal bronchioli (0.4mm in diameter). Only data from the main bronchus are reported herein.

Main bronchus was chosen as the airway of particular interest

in this study for 3 reasons: (i) there are similarities in both size (2-3 mm in diameter) and structure with human small airways (a cartilagenous airway lined by a single layer of cuboidal/low columnar pseudostratified epithelium); (ii) it was considered important to be able to compare the findings with previous studies of TS-induced changes in airway epithelium which have examined this airway in some detail and found it to be a site which responds well to TS exposure (Jones et al., 1972; Jeffery and Reid, 1981) and (iii) the turnover is normally faster and mitotic index greater in extrapulmonary airways than intrapulmonary bronchioli (Bolduc and Reid, 1976).

Small pieces of ileum were also taken as a method control procedure to assess the effectiveness of ^{3}H-T uptake: the large numbers of proliferating epithelial cells rendered the observation of labelled cells more likely. All blocks were postfixed in 1% osmium tetroxide (in 0.1M sodium cacodylate buffer, EM Scope Ltd., London), dehydrated with increasing concentrations of methanol and embedded in Araldite or Spurr's resin (EM Scope Ltd., London) (see Appendix 4) and orientated so that transverse sections of airway rings could be cut. From each animal, rings of main bronchus were taken and a minimum of 20 'sets' of 3 serial sections each were cut with glass knives (EM Scope Ltd., London) using an LKB III ultramicrotome (LKB, U.K.). 'Sets' composed epithelial cell populations separated by steps of 15 m so that (taking the average diameter of cells as 12 μm) each set sampled a distinct population of surface epithelial cells. The sections were mounted on formvar-covered glass slides and processed for autoradiography (Section 2.4). After incubation and development, the

autoradiographs from Experiments 1-4 were stained with 1% Toluidine Blue (in 1% sodium tetraborate) on a hot-plate at approximately 40°C for 10 min. To prevent possible changes in grain number and position this method was modified and the sections from Experiment 5 were stained with 1% aqueous Toluidine Blue in 2.5% Na_2CO_3 (B.D.H. Chemicals Ltd., Essex) at room temperature for 60 min (Boren et al., 1974).

2.4. AUTORADIOGRAPHY

2.4.1. Methods

All light sensitive procedures were carried out in a darkroom under a Kodak No. 2 Red Safe light (Kodak Ltd. U.K.). The range of humidity and temperature observed during the autoradiographic procedures was 60.8% (SEM ± 1.5%) (range 43-71%) and 19.1°C (SEM ± 0.4°C) (range 18-23°) respectively. The autoradiographic procedures were the same for material from all experiments.

Glass slides (Raymond A. Lamb) were cleaned overnight in concentrated chromic acid, rinsed thoroughly in tap water then distilled water and subsequently stored in methanol. Before application of the 1 μm sections, the slides were dipped in a solution of 0.25% formvar (EM-Scope Ltd., U.K.) in CCl_4, to ensure adherence of the sections to the slide.

Ilford K5 Nuclear Research Emulsion (Ilford Ltd., UK.) was used. Fresh emulsion was melted at 43° C, then diluted 1:2 with

deionized distilled water. The procedure is based on the method of Rogers (1979), which produces an emulsion thickness of approximately 3 m (dependent on temperature of the dark room and the speed of dipping). The diluted emulsion was transferred to a Coplin jar, and maintained at 43°C during the subsequent dipping procedure. The dipping technique was chosen in preference to the use of emulsion on stripping film because the former method was simple and ensured the closest possible contact between the emulsion and section.

Hand held slides were dipped and withdrawn slowly and vertically from the emulsion. Slides which had been dipped were examined under the safe light to ensure that the emulsion cover was even and that no air bubbles were present. The trailing edge of the slide was then blotted on tissue to remove excess emulsion; the slide was placed at an angle turned on its long edge, so that the emulsion continued to drain and dry. Slides were allowed to dry in total darkness in a gentle stream of cool air for 3-4 hours. They were then transferred to light-tight storage boxes (Gallenkamp & Co. Ltd.) containing silica gel and incubated at 4°C for 2 weeks, previously assessed as the optimum incubation time (Section 2.2.2).

Slides were developed according to a standard procedure (Feinendengen, 1968): the developer used was undiluted, freshly prepared Kodak D19 (Kodak Ltd. U.K.); developing time was 3 minutes (with 10 seconds of gentle agitation) at a temperature of 19.5°C (SEM. $\pm$ 0.3°) (range 14-22.5°C); slides were rinsed in distilled water for 1 min and fixed in Amphix diluted 1:3 with distilled H_2O for 15 min. They were then washed first in distilled then in gently

running tap water for 20 min, air dried and stained with 1% Toluidine Blue (Section 2.3). Qualitative observation showed that staining after, rather than prior to dipping and development produced fewer background grains. Each glass slide carried control or treated experimental sections of main bronchus together with 4 sections of ileum, 2 from from a ^{3}H-T injected animal and 2 from an animal without ^{3}H-T. The former ilial sections were used to confirm the efficacy of the autoradiographic procedure and to monitor the development of grain density; the latter were a qualitative indicator of the level of background AR grains.

2.4.2. Control Procedures

Background: Possible changes in background due to positive and negative chemography (ie. the process whereby tissue and chemical factors other than β-emission either increase or decrease the number of AR grains observed) were qualitatively monitored.

In the first autoradiographs produced, several tests of negative chemography (ie. factors which might decrease the number of grains) were carried out by exposing an emulsion-covered slide carrying experimental tissue sections of main bronchus and ileum to light, then incubating it under the same conditions as the experimental slides (Rogers, 1979). There was no apparent reduction of grain density over the tissue sections, indicating that the tissue itself had not affected the number of developed grains. It was therefore assumed that the grain density observed over the experimental sections was representative of β-emission from bound nuclear ^{3}H-T.

During each autoradiographic run, positive chemography (ie. an increase in grain density due to the presence of tissue sections) was monitored by the presence of sections of ileum without ^{3}H-T on the experimental slides. After exposure and development, the grain density over these sections was qualitatively compared to background levels over the experimental sections and in the emulsion layer without sections; no qualitative differences were observed in grain density between the 3 areas.

Effect of post-autoradiograph staining on AR grain number and position: two trials of the effect of post-procedure staining with 1% Toluidine Blue were carried out. Serial sections were prepared for ARs then one only was stained by the usual procedure (Section 2.3), and the number and position of grains over both stained and unstained sections were qualitatively compared using phase microscopy. In the first trial, there was no apparent change in either grain number or position, however another later trial showed that there were fewer AR grains over the stained section. In order to reduce the possibility of negative chemography due to staining at high temperature, staining was thereafter done at room temperature for 1 hour according to the method of Boren et al., 1974b (Section 2.3).

2.5. QUANTITATIVE PROCEDURES

Accurate identification of 6 different cell types according to their morphological characteristics, was possible in 1 μm sections

of airway stained with 1% Toluidine Blue. These cells were: **ciliated, serous, mucous, indeterminate, transitional** and basal cells (Jeffery and Reid, 1975). The morphological characteristics of each cell type and thus the criteria by which they were recognized, are described in Chapter 3, Section 3.3.

The population of each cell type is regarded as existing within a separate compartment. A note of clarification regarding indeterminate and transitional cells is necessary. Indeterminate cells (formerly referred to as intermediate or indifferent cells; Jeffery and Reid, 1975; Breeze and Wheeldon, 1977; McDowell et al., 1978) are columnar with a moderately dense to pale-staining cytoplasm devoid of secretory granules as assessed by light microscopy; this group also includes small numbers of tall basal cells and secretory cells with few granules (McDowell et al., 1983) but at the light microscope level it is not possible to resolve these components. Because of the morphological heterogeneity of the secretory cells observed, particularly in TS-exposed animals, the transitional cell group was created to encompass cells containing both dense (serous) and lucent (mucous) secretory granules in varying proportions, ie. either sero-mucous or muco-serous according to the predominance of serous or mucous granules respectively.

It should be noted that the airway epithelium of rat main bronchus is pseudostratified, ie. although every cell touches the basement membrane, not every cell reaches the lumen. Therefore to obtain a reliable sample, basal cells were only counted if a nucleus was apparent and other cells were only included if a full

profile from basement membrane to lumen was observed.

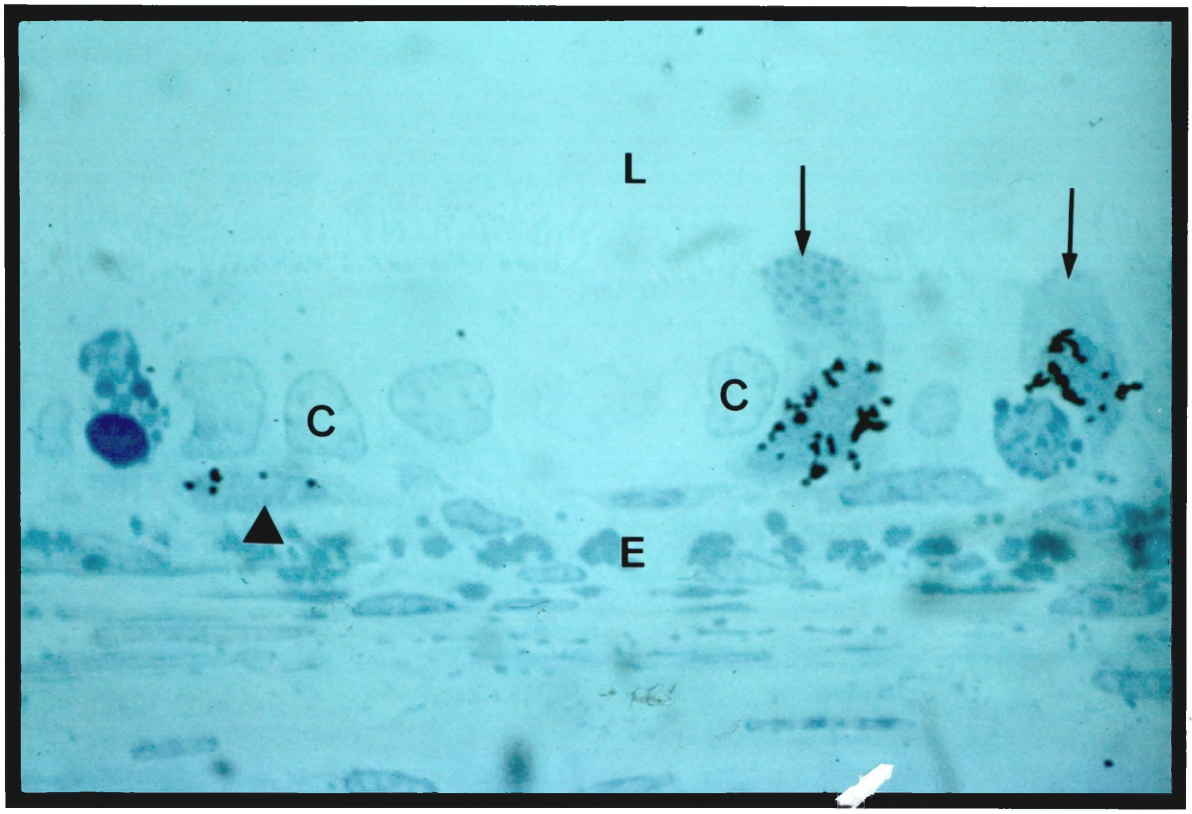
Slides were randomized, coded to avoid observer bias and counted using a Leitz Dialux 20 light microscope fitted with a 'Planapo' Ph. 100X oil immersion objective (Leitz Ltd. U.K.) giving both bright field and phase optics. The best cut and stained section from each set of 3 serial sections was chosen and surface epithelium seen in a maximum of 10 high power (100X oil immersion) fields was quantified over the anterior cartilagenous bronchial wall. The cartilage plate provides physical support ensuring that the dimensions of the epithelium remain as close as possible to the in vivo state throughout the preparative process. The **number of each cell type present** and the **number of each cell type labelled** in epithelium placed across the diameter of each microscopic field was recorded. A cell was considered to be labelled if it showed 4 or more silver grains over the nucleus (Fig 2.2, a & b). It is important to note that the number of grains observed per nucleus was usually larger than 4 and the background levels found during the use of Ilford K5 emulsion were low. The field diameter as measured with a slide micrometer was 0.13 mm and gave a measure of epithelial length. In this way a minimum of 10 and a maximum of 25 step sections (ie. a range of approximately 2600 to 6000 cells) were counted for each animal. From these counts the following ratios were calculated:

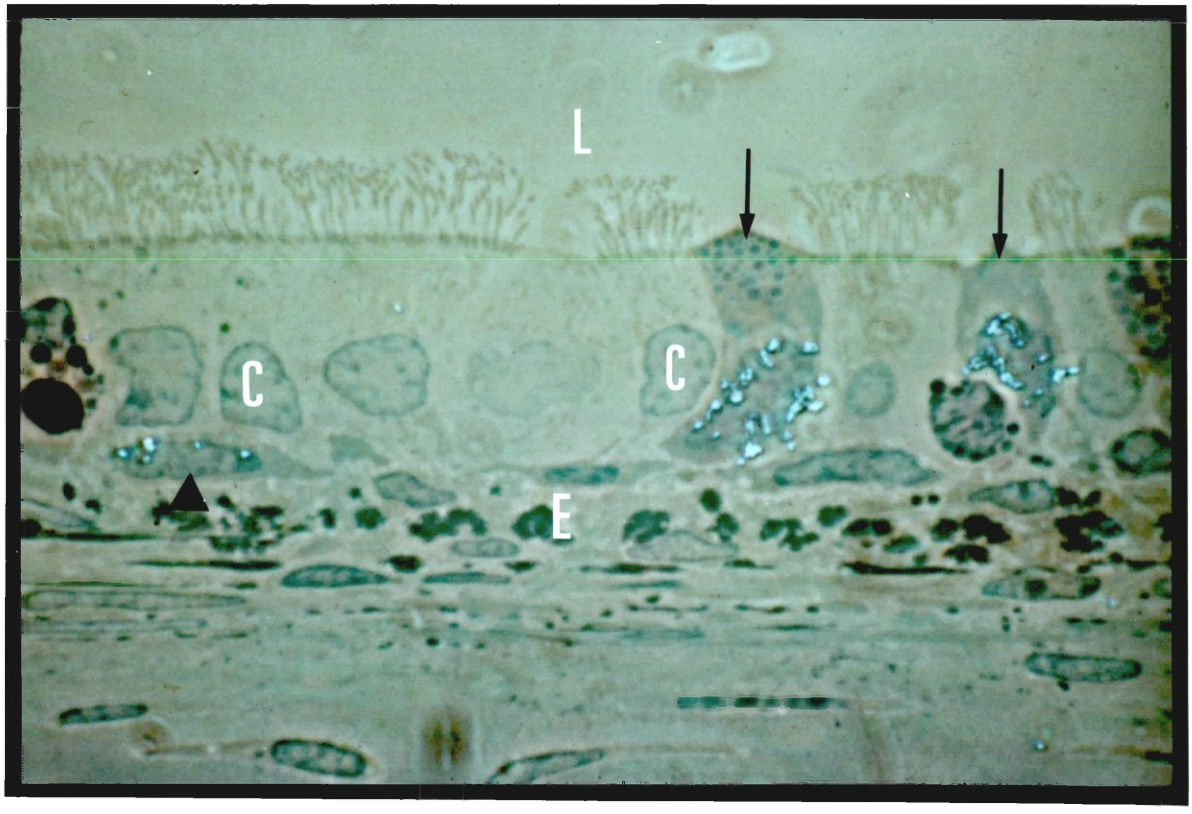
CELLS NUMBERS - CONCENTRATION AND PROPORTIONS

cell concentration = total cell number/10 HPF

Figure 2.2(a) Control epithelium: rat main bronchus. 1μ section stained with Toluidine Blue, dipped in Ilford K5 nuclear emulsion, showing 2 labelled Serous cells (arrows) and 1 labelled Basal cell (arrowhead). Nuclei of Ciliated cells (C), lumen (L), densely-stained elastin (E).
(X 1500, Bright field optics)

Figure 2.2(b) Control epithelium: rat main bronchus. The same field as shown in (a), but photographed with phase optics to show more cellular detail.
(X 1500, Phase optics)


L
C
C
E


L
C
C
E

cell concentration = number cell type/10 HPF
(each cell type)

cell proportion = (number cell type/total cells) %
(each cell type)

LABELLED CELLS - CONCENTRATION AND PROPORTIONS

Each variable was separately determined from both the proliferation and differentiation experiments.

labelled cell concentration = total labelled cell number/10 HPF

labelled cell concentration = number labelled cell type/10 HPF

labelling index = (total labelled cell/total cells) %

labelling index (cell type) = (number labelled cell type/cell type) %

% LABEL DISTRIBUTION

(labelled cell type/total labelled cells) %

The three most important variables to be discussed in ensueing pages, in the context of both proliferation and differentiation experiments, are:

1. Total number of labelled cells per unit length (10 HPF)
2. Number of each cell type labelled/10 HPF
3. % of each cell type labelled
4. % label distribution

The information provided by each variable is clarified in Table 2.5.

TABLE 2.5

EXPLANATION OF QUANTITATIVE VARIABLES

INFORMATION GAINED

Variable	Proliferation Experiments (1, 2 and 5)	Differentiation Experiments (3, 4 and 5)
Total number of labelled cells	Index of epithelial proliferation	Monitors loss of label and cells from epithelium with time
Number of each cell type labelled	Monitors proliferative activity within cell group or compartment	Monitors 'passage' of label from one compartment to another
% Label distribution	Monitors relative contribution of each cell type to proliferative burst	Monitors pathways of differentiation (when considered in conjunction with proliferation data)

2.6. STATISTICAL ANALYSIS

In consultation with Miss Margaret Rehahn (Group Statistician, Brompton Hospital, now Statistical Advisory Services, Sherbourne, Dorset) the distribution of the data was determined. This enabled the application of appropriate statistical tests by the use of software programs written and installed by Miss M. Rehahn for a Prime 750 'Main Frame' computer.

The data distribution of total cell number, number of each cell type, total labelled cell number, number of each labelled cell type were determined for both control and experimental groups. Data for counts of all epithelial cell numbers were normally distributed. Exceptions were found when a particular cell type was present in very small numbers, as often occurred with the mucous cell population; on these occasions distributions were of the Poisson type. Counts of labelled cells followed either a Poisson or a Multiple Poisson distribution, therefore non-parametric statistical tests were used for comparison of median values. These were the Kruskal-Wallis one way analysis of variance by ranks, to test whether all the selected sub-groups were drawn from the same population and the Mann-Whitney U test for the significance of difference between medians of 2 samples.

All data is tabulated as mean values and standard errors (SEM). The basic unit of analysis (n) was a section; thus each mean results from counts of all sections (10 to 25 per animal) from

TABLE 2.6

ERROR FROM REPEAT COUNTS

Rat No.	Section No.	No. of Counts	STANDARD ERROR AS % OF MEAN						
			Ciliated	Serous	Mucous	Transitional	Indeterminate	Basal	Total
1	1	3	0.9	-	1.5	-	1.9	5.1	0.6
	2	3	4.4	-	2.4	-	4.4	4.9	1.7
	3	5	1.5	-	1.2	-	5.2	5.1	0.7
	4	2	3.7	-	2.9	-	24.0	4.9	2.6
2	1	2	3.6	-	20.7	50.5	28.9	1.1	0.5
	2	2	0.4	-	3.1	-	33.6	5.7	2.0
3	1	3	3.6	11.8	-	-	6.4	22.2	7.0
	2	3	4.2	4.0	-	-	23.3	6.9	2.6
	3	4	2.7	5.5	-	-	22.7	5.8	3.2
4	1	2	0.7	12.2	50	-	12.1	10.2	5.4
	2	2	6.2	-	-	-	11.2	5.1	1.5
5	1	3	1.0	8.8	-	33.3	12.5	4.4	0.8
	2	4	1.9	2.3	38.2	34.4	16	1.6	3.2
6	1	2	1.2	6.2	-	7.8	2.6	3.7	3.2
	2	2	2.2	0.8	-	-	2.6	2.2	1.8
Total mean % error			2.6	3.4	8.0	8.4	13.8	5.9	2.5
Standard error			± 0.4	± 1.2	± 4.1	± 4.3	± 2.7	± 1.3	± 0.5

animals at each time point.

Body weight data were normally distributed.

P values of less than or equal to 0.05 (the 5% level) were taken as the acceptable level of statistical significance for differences between mean or median values.

2.7. DETERMINATION OF FACTORS CONTRIBUTING TO VARIATION IN THE COUNT

The variation within and between animals was determined. The variation between two different observers was not assessed.

2.7.1. Observer Error

Data from repeat counts done throughout the study was compiled: 6 rats from both control and TS-exposed groups were used and from each rat all cell types were counted in 2-4 sections; each section was counted 2-5 times. The mean, SEM, % variation - ie. (SEM/mean)%, were determined for each set of repeat counts. Subsequently, the Mean % variation and corresponding SEM were determined from these data (Table 2.6).

For both individual sections and the totalled data, variation was greatest for cell types which were infrequent ie. mucous, transitional and indeterminate groups. The overall Mean % Error for ciliated cells was 2.6% (± 0.43), for serous cells 3.4% (± 1.2),

TABLE 2.7

INTRA-ANIMAL VARIATION - BETWEEN SECTIONS

Group of Sections	No. of Sections	STANDARD ERROR AS % OF MEAN						
		Ciliated	Serous	Mucous	Transitional	Indeterminate	Basal	Total
1	30	3.8	7.4	32.0	21.1	7.7	3.0	1.9
2	30	4.0	7.1	38.7	16.3	8.8	3.3	1.5

TABLE 2.8

INTRA-ANIMAL VARIATION - BETWEEN ANIMALS

STANDARD ERROR AS % OF MEAN						
Ciliated	Serous	Mucous	Transitional	Indeterminate	Basal	Total
4.2	9.4	32.7	19.9	9.3	3.2	2.3

mucous cells 8.0% (± 4.1), transitional cells 8.4% (± 4.3), indeterminate cells 13.8% (± 0.7), basal cells 5.9% (± 1.3) and total cells 2.5% (± 0.5).

2.7.2. Intra-animal Variation

To determine error within animals due to variation between sections, the mean, SEM and % variation were calculated from the first five sections (each containing 10 fields) from one animal from each time point in control and smoked proliferation groups (a total of 30 sections). These calculations were repeated for another five-section group (30 sections) from the same animals; Table 2.7 shows that there was little difference between the two groups. As with observer error, the greatest variation within animals was found for cell types which are low in number.

2.7.3. Inter-animal Variation

The inter-animal mean, SEM and % variation were determined by comparing counts on one section from each of two animals at every time point from the 4 experimental groups (a total of 31 sections from control, smoked proliferation, control differentiation and smoked differentiation); again, the highest variation was observed in the transitional and mucous cell groups (Table 2.8).

2.7.4. Total Percentage variation

The mean % variation incurred during counting of airway epithelial cells in this study, from all sources is: ciliated cells

3.6%, serous cells 6.8%, transitional cells 11.6%, mucous cells 27.9%, indeterminate cells 9.9%, basal cells 3.9% and total cells 2.1%.

The extent of the variation shows a consistent pattern: mucous cells make the largest contribution, followed by transitional then indeterminate cells. Mucous cells are infrequent, particularly in control animals and have been shown to follow a Multiple Poisson distribution (Section 2.6) which suggests considerable variation in numbers between animals.

CHAPTER 3 – NORMAL AIRWAY EPITHELIUM

3.1. INTRODUCTION

Chapter 3 presents results from experiments which investigate proliferation and differentiation in main bronchial epithelium from control SPF rats (Experiments 2, 3 and 4; see Tables 2.1 and 2.2). The experimental methods are described in detail in Chapter 2.

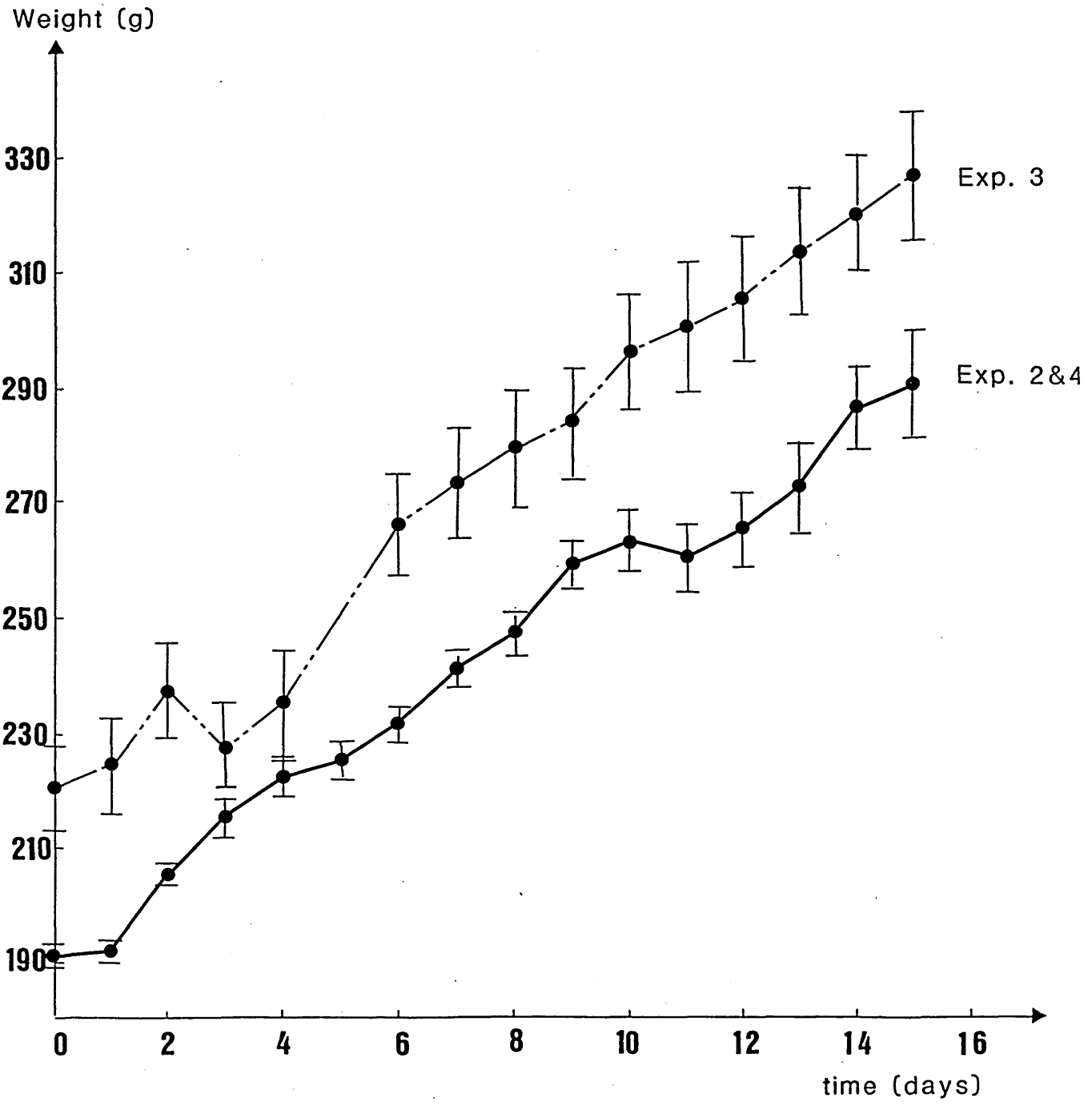
For studies of proliferation, 10 control rats were killed 1 h after ^{3}H-T. 1 μm plastic sections of main bronchus and ileum were cut, autoradiographed and quantitative analysis of main bronchus was undertaken (see Chapter 2, Sections 2.3, 2.4 and 2.5). For studies of differentiation, 19 control animals were given a single pulse of ^{3}H-T on day 0 and subsequently killed on 1, 3, 7, 10 and 14 days. Samples of main bronchus and ileum were removed, processed, autoradiographed and analysed as described in Chapter 2.

3.2. ANIMAL WEIGHTS

The weights of control animals from concurrent experiments (2&4 combined) and of those from Experiment 3 are displayed in Fig 3.1. The graph shows that all control animals from both proliferation and differentiation studies gained weight during the experimental period.

At day 14 there was no significant difference between the mean weights of animals in the proliferation and differentiation groups in Experiments 2&4. Significant differences existed between the

FIGURE 3.1 CONTROL ANIMAL WEIGHTS



mean weights of the combined Experiments 2&4 and Experiment 3 at the beginning and end of the experimental period, but their rates of weight increase were equivalent.

3.3. CELL TYPES: NUMBER AND PROPORTION

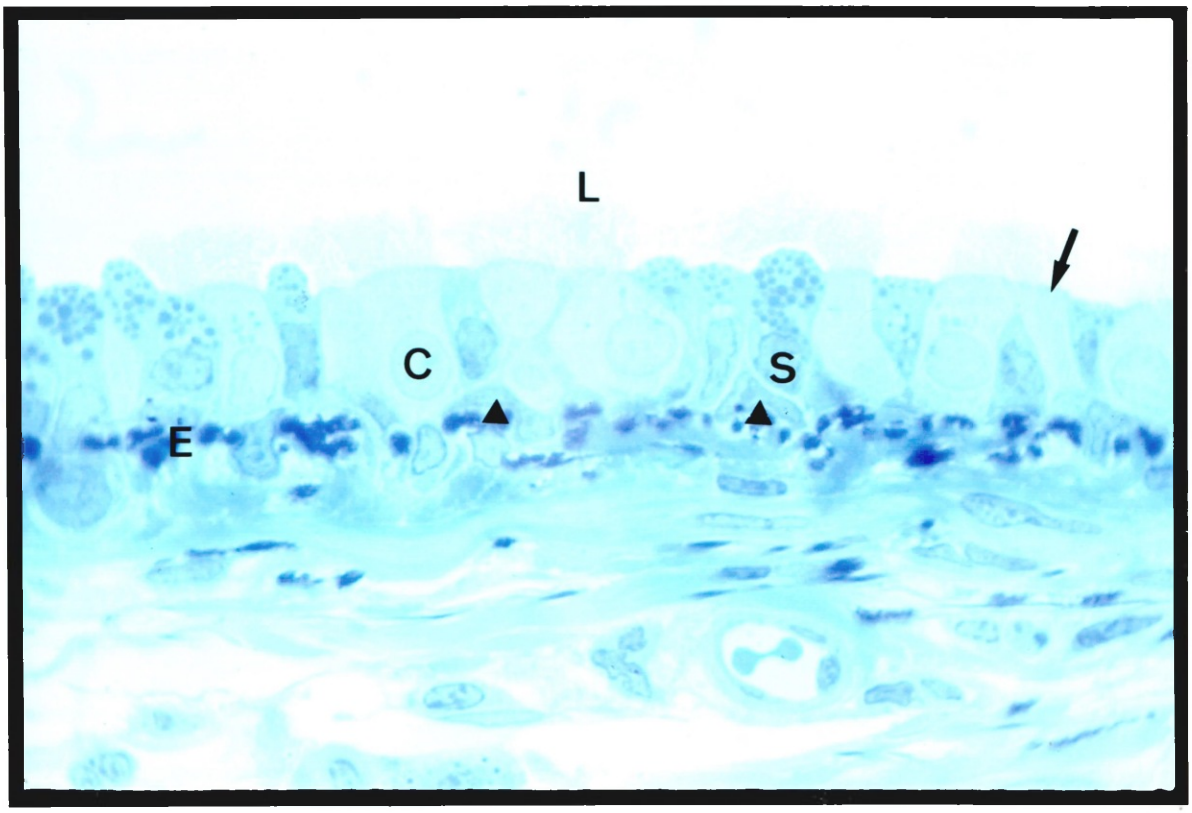
The anterior wall of the main bronchus in both proliferation and differentiation control animals was lined by pseudostratified columnar epithelium (Fig 3.2). In 1 μm sections stained with Toluidine blue it comprised ciliated cells, secretory cells made up of serous, mucous and transitional cells (see Chapter 2, Section 2.5) a non-secretory group of indeterminate morphology (formerly referred to as 'intermediate' or 'indifferent' cells (Jeffery and Reid, 1975; Breeze and Wheeldon, 1977; McDowell et al., 1978) and basal cells (Fig 3.2). Intra-epithelial globular leukocytes and lymphocytes were also observed but were not counted.

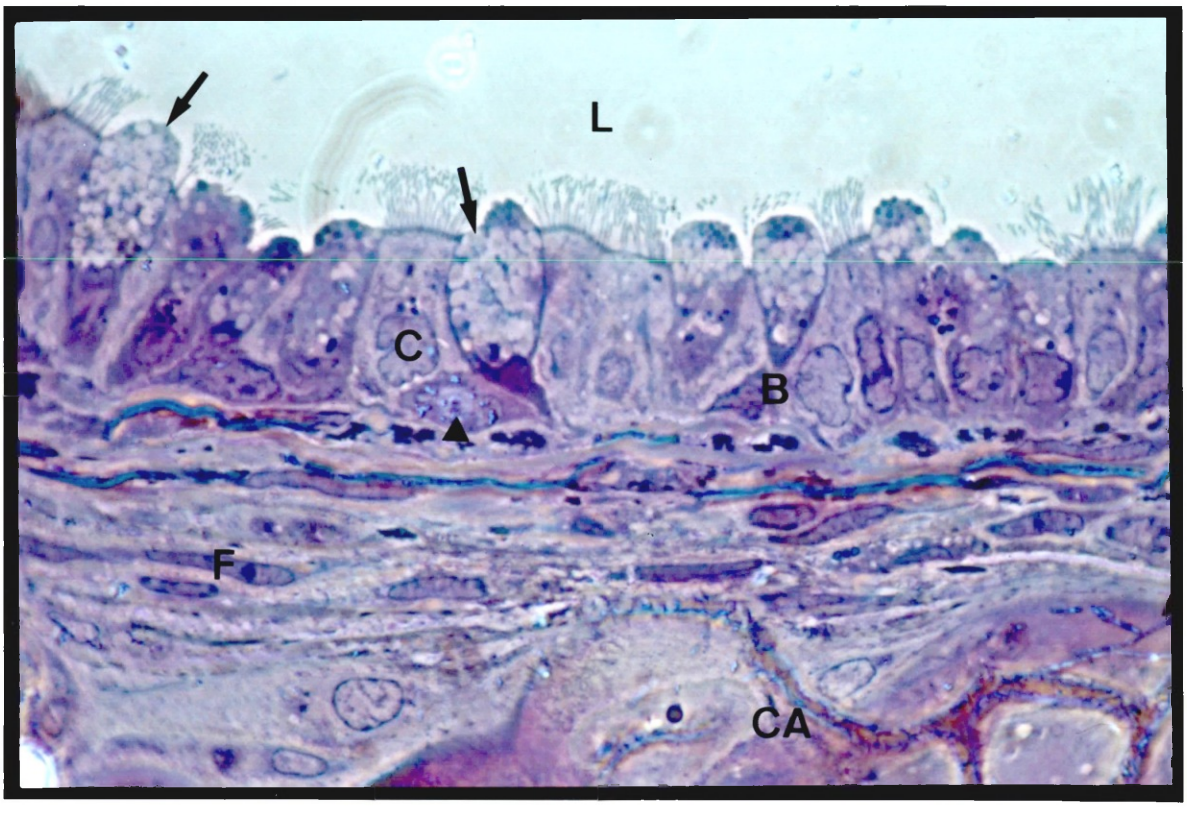
Ciliated cells extended from the basement membrane to the airway lumen with a pale (ie. poorly stained) cytoplasm containing a variable number of densely stained lysosomes. The apical luminal border was lined by densely stained basal bodies from which cilia extended into the lumen. The basally positioned nucleus was round to oval in shape with sparse, peripherally arranged heterochromatin (Fig 3.2).

The three types of **secretory cell** were similar in general morphology but for their type of secretory granule. Each cell spanned the full depth of the epithelium; the cytoplasm was moderately stained and the nuclei basally situated, oval or

Figure 3.2 Control epithelium: rat main bronchus.
1μ section stained with Toluidine Blue, showing Ciliated cells (C), Serous cells (S), an Indeterminate cell (arrow) and Basal cells (arrowheads), Lumen (L), Elastin (E).
(X 300, Bright field optics)

Figure 3.3 14 day TS-exposed epithelium: rat main bronchus.
1μ section stained with Toluidine Blue showing Ciliated cells (C), Mucous-secreting cells (arrows) and Basal cells (B) one of which is labelled (arrowhead). Lumen (L), cartilage (CA), fibroblast (F).
(X 1300, Phase optics)


L
C
S
E


L
C
B
F
CA

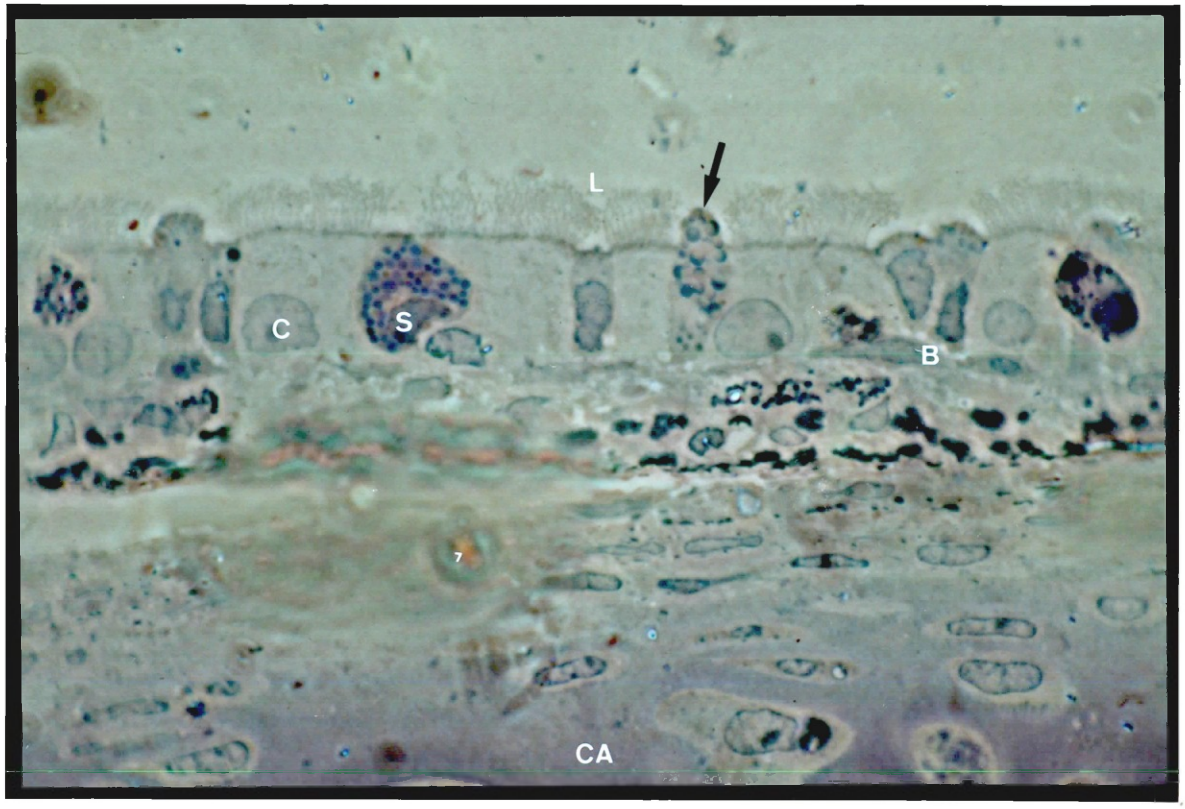
indented with scattered chromatin clumps and prominent nucleoli; nuclear staining was dense. The cytoplasm contained varying numbers of secretory granules: **serous** cells were counted when they contained 4 or more discrete, round, regular and densely stained granules: when few in number they were present at the cell apex. **Mucous** cells were rarely observed in control SPF animals, but present in animals exposed to TS; they contained round, weakly stained or translucent granules in apposition to each other (Fig 3.3). **Transitional** cells contained a mixture of both serous and mucous granules and occasionally individual granules with a mixture of dense and lucent material (Fig 3.4). This cell type was counted as 2 groups, sero-mucous and muco-serous, according to which granule type was predominant. For presentation herein, the counts were combined.

Indeterminate cells, which spanned the depth of the epithelium, had a moderately stained homogeneous cytoplasm, an oval nucleus, and no secretory granules detectable by light microscopy (Fig 3.2). Cells showing a brush border (ie. Brush cells) were included in this category.

Basal cells were roughly triangular or elongate in shape, usually when they were orientated with their long axes parallel to the basement membrane. The densely stained cytoplasm was sparse and the nuclei, also densely stained, had prominent nucleoli (Fig 3.2).

Tables 3.1 and 3.2 give the cell number data from the unexposed animals. Counts of the **Total Number** of epithelial cells and the **Number of Each Cell Type** are expressed per unit length of

FIGURE 3.4 Control epithelium: rat main bronchus. 1μ section stained with Toluidine Blue showing Ciliated cells (C), a Serous cell (S) and a Transitional cell (arrow) with granules containing both dense and lucent material. Basal cells (B), Lumen (L), Cartilage (CA). (X 1300, Phase optics)


L
C
S
B
CA

epithelium (ie. 10 high power fields or 0.13 mm length) or as percentages of all epithelial cells counted. Based on counts in the three experiments on day 0 there were on average 1830 (SEM ± 15) and on day 14, 1813 (SEM ± 59, range 1708-1908) cells per mm in the main bronchial epithelium of control rats (Table 3.1).

Taking the 10 'proliferation' control rats, **ciliated** cells were the most prevalent at 43% of the population, **serous** comprised 24%, **mucous** 0.3%, **transitional** 3.0%, **indeterminate** 8% and **basal** cells 22% of the population (Table 3.2).

Examination of data from the beginning and end of each experiment reveals small significant changes in the cell counts during the 14 day period. In Experiments 2&4, (experiments carried out concurrently) total cell numbers decreased by a small amount, ciliated and mucous cells remained unchanged, and serous cells decreased; transitional and basal cells decreased and indeterminate cells increased slightly (Table 3.1). Experiment 3, carried out at an earlier time, showed similar but more pronounced changes. In particular, mucous cells increased in number. These data expressed as percentages show significant changes in the distribution of several cell types in Experiments 2&4 and 3 (Table 3.2).

3.4. PROLIFERATION

Counts of labelled cells in the main bronchial epithelium of unexposed animals (Experiment 2 only) are presented as: 1) labelled cell number per unit length epithelium, 2) the labelled proportion

TABLE 3.1

CONTROL

Epithelial Cell Numbers/10 HPF (± SEM)

CELL TYPE	Time (days) 0	1	3	7	10	14
CILIATED						
Exp 2&4	103 (±1.8)	96.1 (±1.6)	106.2 (±1.8)	105.2 (±2.4)	115.5 (±4.1)	101.6 (±1.8) NS
Exp 3			108.5 (±2.6)			126.5 (±2.4)***
SEROUS						
Exp 2&4	56.9 (±1.4)	58.3 (±2.0)	32.4 (±2.4)	65.0 (±1.5)	37.4 (±3.5)	49.5 (±1.4)***
Exp 3			53.4 (±2.5)			22.7 (±2.5)***
MUCOUS						
Exp 2&4	0.7 (±0.2)	0.0	9.1 (±1.2)	1.8 (±0.3)	8.7 (±1.2)	0.96 (±0.1)*
Exp 3			2.8 (±0.6)			23.3 (±3.4)***
TRANS-ITIONAL						
Exp 2&4	7.2 (±0.2)	0.05 (±0.03)	12.23 (±0.7)	4.4 (±0.3)	16.5 (±0.8)	4.4 (±0.2)***
Exp 3			5.6 (±0.4)			4.4 (±0.3) NS
INDETER-MINATE						
Exp 2&4	18.9 (±0.7)	22.0 (±1.3)	36.8 (±1.7)	19.5 (±0.8)	16.0 (±1.1)	24.4 (±0.8)***
Exp 3			20.7 (±1.5)			21.9 (±1.6) NS
BASAL						
Exp 2&4	51.4 (±0.6)	46.6 (±1.7)	50.5 (±1.3)	54.2 (±0.8)	55.7 (±0.7)	48.1 (±0.6)*
Exp 3			53.6 (±1.4)			48.5 (±1.4)**
TOTAL						
Exp 2&4	238.4 (±1.9)	224.7 (±3.6)	246.5 (±3.6)	250. 0 (±2.8)	250.7 (±2.0)	229.5 (±2.0)**
Exp 3			251.5 (±3.1)			248.4 (±2.1) NS

NS Not significantly different
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

} compared to day 0 (Exp 2&4)
day 3 (Exp 3)

TABLE 3.2

CONTROL

% Epithelial Cell Type (± SEM)

CELL TYPE	Time (days) 0	1	3	7	10	14
CILIATED						
Exp 2&4	43.2 (±0.5)	42.8 (±0.7)	43.1 (±0.4)	41.9 (±0.6)	45.9 (±1.6)	44.0 (±0.7) NS
Exp 3			44.3 (±0.6)			50.9 (±0.8)***
SEROUS						
Exp 2&4	24.0 (±0.6)	25.9 (±0.8)	13.3 (±1.0)	26.1 (±0.6)	15.1 (±1.5)	22.0 (±0.8) NS
Exp 3			22.0 (±1.1)			9.1 (±1.0)***
MUCOUS						
Exp 2&4	0.3 (±0.06)	0.0	3.5 (±0.4)	0.7 (±0.1)	3.5 (±0.5)	0.4 (±0.06)*
Exp 3			1.2 (±0.3)			9.4 (±1.4)***
TRANS-ITIONAL						
Exp 2&4	3.1 (±0.1)	0.023 (±0.02)	4.7 (±0.2)	1.7 (±0.1)	6.6 (±0.2)	1.9 (±0.1)***
Exp 3			2.4 (±0.2)			1.8 (±0.1)*
INDETER-MINATE						
Exp 2&4	8.0 (±0.3)	9.8 (±0.5)	15.4 (±0.8)	7.9 (±0.4)	6.4 (±0.4)	10.7 (±0.3)***
Exp 3			8.4 (±0.6)			8.8 (±0.6) NS
BASAL						
Exp 2&4	21.6 (±0.2)	21.6 (±0.6)	20.4 (±0.3)	21.7 (±0.3)	22.2 (±0.2)	21.0 (±0.2) NS
Exp 3			21.8 (±0.3)			19.5 (±0.6)***

NS Not significantly different
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

compared to day 0 (Exp 2&4)
day 3 (Exp 3)

of each cell compartment, expressed as a percentage (Table 3.3), and 3) the percentage contribution of each labelled cell type to the total labelled cell population (Table 3.4).

3.4.1. Total number of labelled cells

Table 3.3 shows that on day 0, about 1 labelled cell per unit length (10 HPF, 0.13mm) was found in normal rat main bronchial epithelium, representing about 0.4% of the population (ie. 1 cell/220 cells counted). After 14 days this value had fallen to about 0.2% of the population (ie. the ratios were 1 cell/0.26 mm or 1 cell/550 cells counted).

3.4.2. Number of each cell type labelled

Table 3.3 shows that all cells except mucous cells normally contribute to the proliferating population (ie. the growth fraction). Mucous cells were infrequently found in unexposed epithelium and were never found labelled. The proportion of each compartment that was labelled tended to decrease during the 2 weeks of the experiment, significantly so for basal (p 0.01) and serous ($p<0.05$) cells. On day 0, 1 in 68 basal cells was labelled but by day 14 the ratio was 1/152. On day 0, 1 in 316 serous cells was labelled, falling by day 14 to 1 in 1200. (These ratios were calculated from data in Tables 3.3 and 3.1).

3.4.3. Percentage label distribution

When the numbers of each labelled cell type were expressed as

TABLE 3.3

CONTROL PROLIFERATION - EXPERIMENT 2

	Labelled Epithelial Cells/ 10 HPF (± SEM) Time (days)		(Labelled Cells/Cell type) %	
	0	14	0	14
CILIATED	0.02 (± 0.01)	0.0 NS	0.02 (± 0.01)	0 NS
SEROUS	0.18 (± 0.04)	0.05 (± 0.03)*	0.33 (± 0.07)	0.11 (± 0.07)*
MUCOUS	0	0	0	0
TRANSITIONAL	0.015 (± 0.01)	0 NS	0.12 (± 0.06)	0 NS
INDETERMINATE	0.1 (± 0.03)	0.03 (± 0.02) NS	0.56 (± 0.2)	0.20 (± 0.15) NS
BASAL	0.75 (± 0.09)	0.31 (± 0.07)**	1.5 (± 0.8)	0.66 (± 0.16)**
TOTAL	1.06 (± 0.1)	0.40 (± 0.09)***	0.45 (± 0.05)	0.18 (± 0.04)**

NS Not significant
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

} compared to day 0 values

TABLE 3.4

CONTROL PROLIFERATION - EXPERIMENT 2

% LABEL DISTRIBUTION

CELL TYPE	Time (days) 0	14
CILIATED	2	0
SEROUS	17	13
MUCOUS	0	0
TRANSITIONAL	2	0
INDETERMINATE	9	8
BASAL	71	78

a percentage of all labelled cells, the major contributors to the proliferating fraction were basal, serous and indeterminate cells. There were small changes in their proportions with time (Table 3.4).

3.4.4. Circadian Variation

Control animals killed in the morning (0800hr) showed nearly twice as many labelled cells per unit length epithelium as those killed during the evening (20.00 hr) ($p<0.05$) (Table 3.5). The difference was due to a decrease in the numbers of labelled serous cells (Table 3.6).

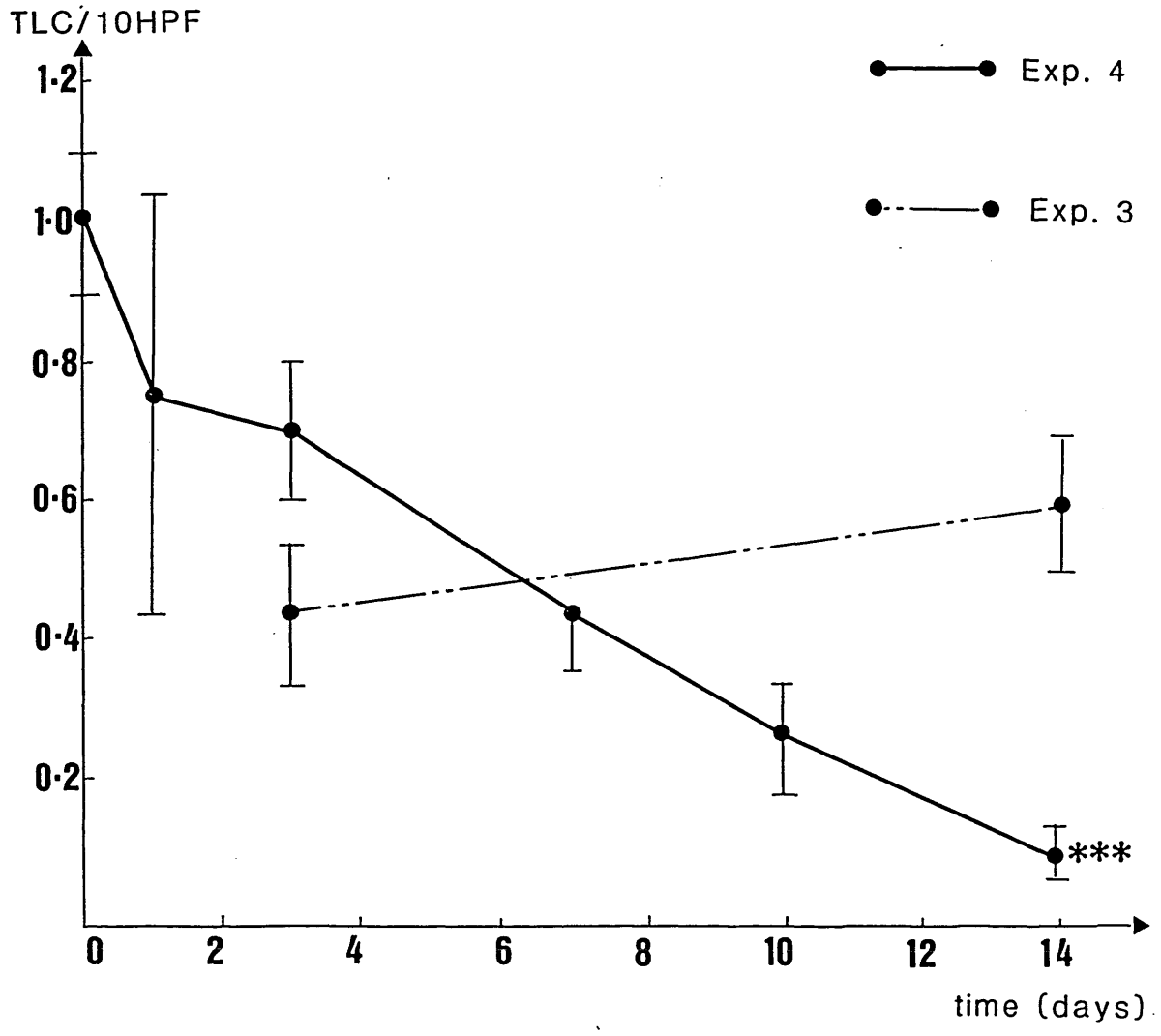
3.5. DIFFERENTIATION

3.5.1. Total number of labelled cells

The following data from Experiments 3 and 4, (carried out at different times, see Table 2.2) represent labelled cells resulting from a single pulse of ^{3}H-T administered at the beginning of the experiment only and taken up by a cohort of actively dividing cells. Subsequent changes with time in the total number of labelled cells and changes in the distribution of label between cell types, were assessed for both experiments and used to monitor pathways of differentiation (Table 3.7).

The data from Experiment 4 shows that on day 0, one cell per unit length epithelium, ie. 0.4% of cells present, was labelled. Fig. 3.5 shows that the **total number of cells labelled** per 10 HPF

FIGURE 3.5 CONTROL DIFFERENTIATION:
TOTAL LABELLED EPITHELIAL CELLS/10 HPF VS TIME (± SEM)



* p < 0.05
** p < 0.01
*** p < 0.001

} compared to day 0 (Exp. 4)
day 3 (Exp. 3)

TABLE 3.5

CONTROL PROLIFERATION - EXPERIMENT 2

Morning and Evening

Time	Total Labelled Epithelial Cells/ 10 HPF (± SEM)	(Labelled Cells/ Total Cells) (± SEM) %
8:00 (n = 3)	1.4 (± 0.2)	0.62 (± 0.1)
20:00 (n = 4)	*0.86 (± 0.1)	**0.35 (± 0.05)

* $p < 0.05$

** $p < 0.01$

} compared to 8:00 values

TABLE 3.6

CONTROL PROLIFERATION - EXPERIMENT 2

Morning and Evening

CELL TYPE	Time	Labelled Cells/ 10HPF (± SEM)	Labelled Cells/ Cell Type) %
CILIATED	8:00	0.02 (± 0.02)	0.03 (± 0.03)
	20:00	0.01 NS (± 0.01)	0.01 NS (± 0.01)
SEROUS	8:00	0.31 (± 0.07)	0.6 (± 0.1)
	20:00	0.1** (± 0.03)	0.2** (± 0.06)
MUCOUS	8:00	0	0
	20:00	0	0
TRANSITIONAL	8:00	0.041 (± 0.03)	0.3 (± 0.2)
	20:00	0 NS	0 NS
INDETERMINATE	8:00	0.17 (± 0.07)	1.1 (± 0.6)
	20:00	0.06 NS (± 0.03)	0.25 NS (± 0.1)
BASAL	8:00	0.8 (± 0.1)	1.8 (± 0.3)
	20:00	0.7 NS (± 0.1)	1.3 NS (± 0.2)

NS Not significant

** $p < 0.01$

} compared to 8:00 values

decreases steadily during the experimental period, to become significantly lower by 7 days ($p<0.001$); by 14 days the value was 10% of the original figure. This observation contrasts with the findings in Experiment 3 where there was no significant difference between the number of cells labelled per unit length on days 3 and 14.

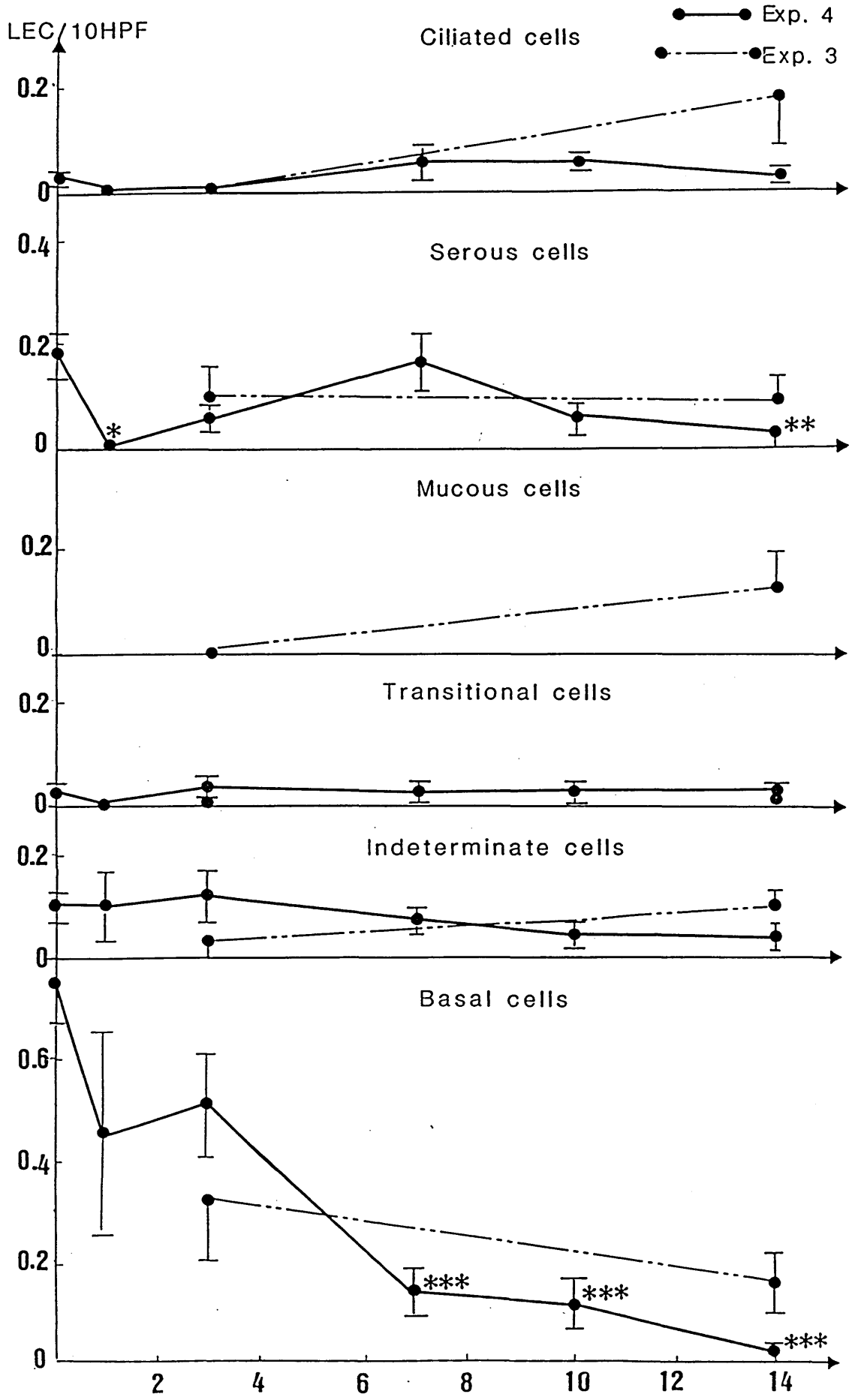
3.5.2. Number of each cell type labelled

Fig. 3.6 and Table 3.7 show changes in the numbers of each labelled cell type per unit length epithelium with time, in both experiments. In Experiment 4, significant changes were observed only in the labelled serous and basal cell compartments. Initially labelled serous cells followed a fluctuating course, decreasing significantly to low levels ($p<0.05$) but then rising to the starting value at 7 days before a further decrease at 14 days ($p<0.01$). The number of labelled basal cells showed a steady decrease which became statistically significant at day 7 ($p<0.001$); the value at 14 days remained significantly less than the control day 0 level ($p<0.001$). In Experiment 3, although no cell compartment showed statistically significant change, labelled mucous cells were observed for the first time at 14 days.

3.5.3. Percentage label distribution

Trends within the cohort of labelled cells became clearer when the number of each labelled cell type was expressed as a percentage of the total labelled cell population (Table 3.8). In both experiments, the most striking shifts were observed in the

FIGURE 3.6 CONTROL DIFFERENTIATION:
LABELLED EPITHELIAL CELLS/10 HPF VS TIME (± SEM)



* p < 0.05
** p < 0.01
*** p < 0.001

compared to day 0 (Exp. 4)
day 3 (Exp. 3)

TABLE 3.7

CONTROL DIFFERENTIATION - EXPERIMENTS 4 and 3

Labelled Epithelial Cells /10 HPF (± SEM)

CELL TYPE	Time (days) 0	1	3	7	10	14
CILIATED						
Exp 4	0.02(±0.01)	0	0	0.05(±0.03)	0.05(±0.02)	0.016(±.016) NS
Exp 3			0			0.17(±.09) NS
SEROUS						
Exp 4	0.18(±0.04)	0	0.05(±0.03)	0.16(±.06)	0.05(±0.3)	0.017(±.017)**
Exp 3			0.09(±.06)			0.08(±.05) NS
MUCOUS						
Exp 4	0	0	0	0	0	0
Exp 3			0			0.11(±.07) NS
TRANS-ITIONAL						
Exp 4	0.016(±0.01)	0	0.03(±.02)	0.02(±0.02)	0.02(±.02)	0.02(±.02) NS
Exp 3			0			0
INDETER-MINATE						
Exp 4	0.10(±.03)	0.1(±.07)	0.12(±0.05)	0.07(±.03)	0.04(±.02)	0.03(±.02) NS
Exp 3			0.03(±.03)			0.09(±.05) NS
BASAL						
Exp 4	0.75(±.09)	0.45 (±.2)	0.51(±.1)	0.14(±.05)	0.11(±.05)	0.017(±.017)***
Exp 3			0.32(±.1)			0.15(±.06) NS
TOTAL						
Exp 4	1.06 (±.1)	0.75(±.3)	0.70(±.1)	0.44(±.1)	0.27(±.08)	0.1(±.04)***
Exp 3			0.44(±.1)			0.6(±.1) NS

NS Not significantly different
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

} compared to day 0 (Exp 2 & 4)
day 3 (Exp 3)

TABLE 3.8

CONTROL DIFFERENTIATION

% Label Distribution

Time (days)	CELL TYPE					
	Ciliated	Serous	Mucous	Transitional	Indeterminate	Basal
			Experiment 4			
0	2	17	0	2	9	71
1	0	0	0	0	40	60
3	0	7	0	4	17	73
7	11	36	0	5	16	32
10	19	19	0	7	15	41
14	16	17	0	20	30	17
			Experiment 3			
3	0	20	0	0	7	73
7	-	-	-	-	-	-
14	28	13	18	0	15	25

proportions of the labelled ciliated and basal cell compartments. Over 14 days, the contribution of ciliated cells increased from 2% to 16% and 0 to 28% in Experiments 4 and 3 respectively. By contrast, the basal cell contribution dropped from 71 to 17% and 73 to 25%.

In Experiment 4, labelled mucous cells were absent, but transitional cells changed from 1% (on day 0) to 20% of the total labelled population on day 14. In Experiment 3 there were no labelled transitional cells, but by 14 days mucous cells formed 18% of the labelled cell population.

In Experiment 4, by 24 h, the proportion of labelled cells that were indeterminate had increased concurrent with a decrease in the labelled serous cell proportion. Thereafter, changes in these cell compartments appeared to mirror each other, although the proportion of labelled indeterminate cells was higher at the end than at the beginning of the experiment. In Experiment 3 also, the proportions of labelled serous cells decreased and indeterminate cells increased concurrently.

3.6. SUMMARY

Cell Numbers

1. The data pooled from beginning and end of the experiment showed that in main bronchial epithelium of control, healthy male, Sprague-Dawley, SPF rats (180-220 g): ciliated cells comprised

43%, **serous** cells 24%, **basal** cells 22%, **indeterminate** cells 8%, **transitional** cells 3%, and **mucous** cells 0.3% of the cell population.

2. During the experimental period, the control epithelial cell population from unexposed rats underwent small though statistically significant variations: the total number of cells per unit length epithelium remained unchanged in one experiment and decreased slightly in the other.

Proliferation

3. Experiment 2 showed that after one pulse of ^{3}H-T the proliferating cell population in airway epithelium was comprised of: **basal** cells (71%), **serous** cells (17%), **indeterminate** cells (9%), **transitional** (1%) and **ciliated** cells (2%). These proliferating cells contribute to the growth fraction.

4. The data suggested that a **circadian variation** exists in the proliferation of epithelial cells: significantly more cells proliferated in the morning (0800 hr) than in the evening (20.00 hr) due to a decrease in the numbers of dividing serous cells.

Differentiation

5. During the differentiation experiments (3&4), the total number of cells per unit length epithelium remained relatively

constant. However, the fate of the two cell populations labelled on day 0 differed: the number of labelled cells remained constant from days 3 to 14 in one experiment but decreased to 10% of the original level in the other.

6. In Experiment 4, significant decreases occurred with time in the numbers of the labelled serous and basal cells only and no labelled mucous cells were observed.

7. The percentage label distribution data showed the same trends in both experiments: the proportion of the labelled population that was basal decreased while that which was ciliated and transitional (Exp 4) or mucous (Exp 3) increased steadily with time; changes in labelled indeterminate and serous cell proportions mirrored each other.

Interpretation of these changes is discussed in Chapter 6.

CHAPTER 4 – CHANGES INDUCED BY TOBACCO SMOKE

4.1. INTRODUCTION

Chapter 4 presents the results of experiments which examine the effect of Tobacco Smoke (TS) on proliferation (see Table 2.1; Experiments 1&2) and differentiation (see Table 2.2; Experiments 3&4) in SPF rat main bronchial epithelium. The experimental methods used are described in Chapter 2. The animal weight and cell number data from Experiments 1&3 have been combined, as have the data from Experiments 2&4 (Tables 4.1, 4.2 and 4.3); both these experimental sets were conducted concurrently.

4.2. ANIMAL WEIGHTS

Fig. 4.1 (a&b) shows that although all animals used in these experiments gained weight, there were significant differences between control and TS-exposed groups: the difference was greater in Experiments 1&3 than Experiments 2&4.

4.3. CELL TYPES: NUMBER AND PROPORTIONS

As a result of exposure to TS, airway epithelium showed three qualitative morphological changes: it was readily observable that the number of mucous cells had increased, serous cells had decreased and the width of TS-exposed epithelium was greater than that of controls (Fig 4.2 a&b); these changes were more pronounced in Experiments 1&3.

The total cell number and the number of each cell type per

FIGURE 4.1(A) ANIMAL WEIGHTS - EXPERIMENTS 1 & 3

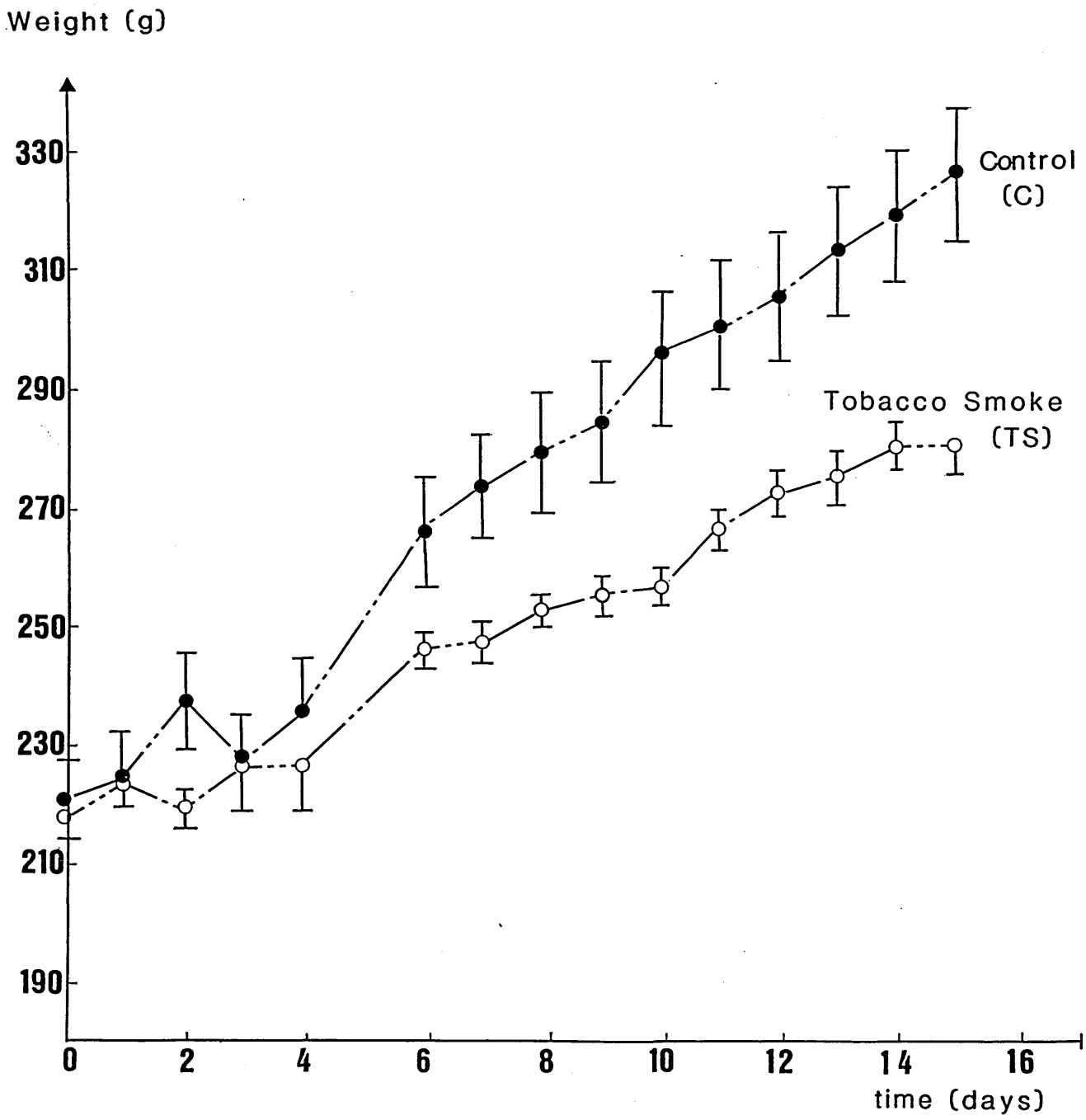


FIGURE 4.1(B) ANIMAL WEIGHTS - EXPERIMENTS 2 & 4

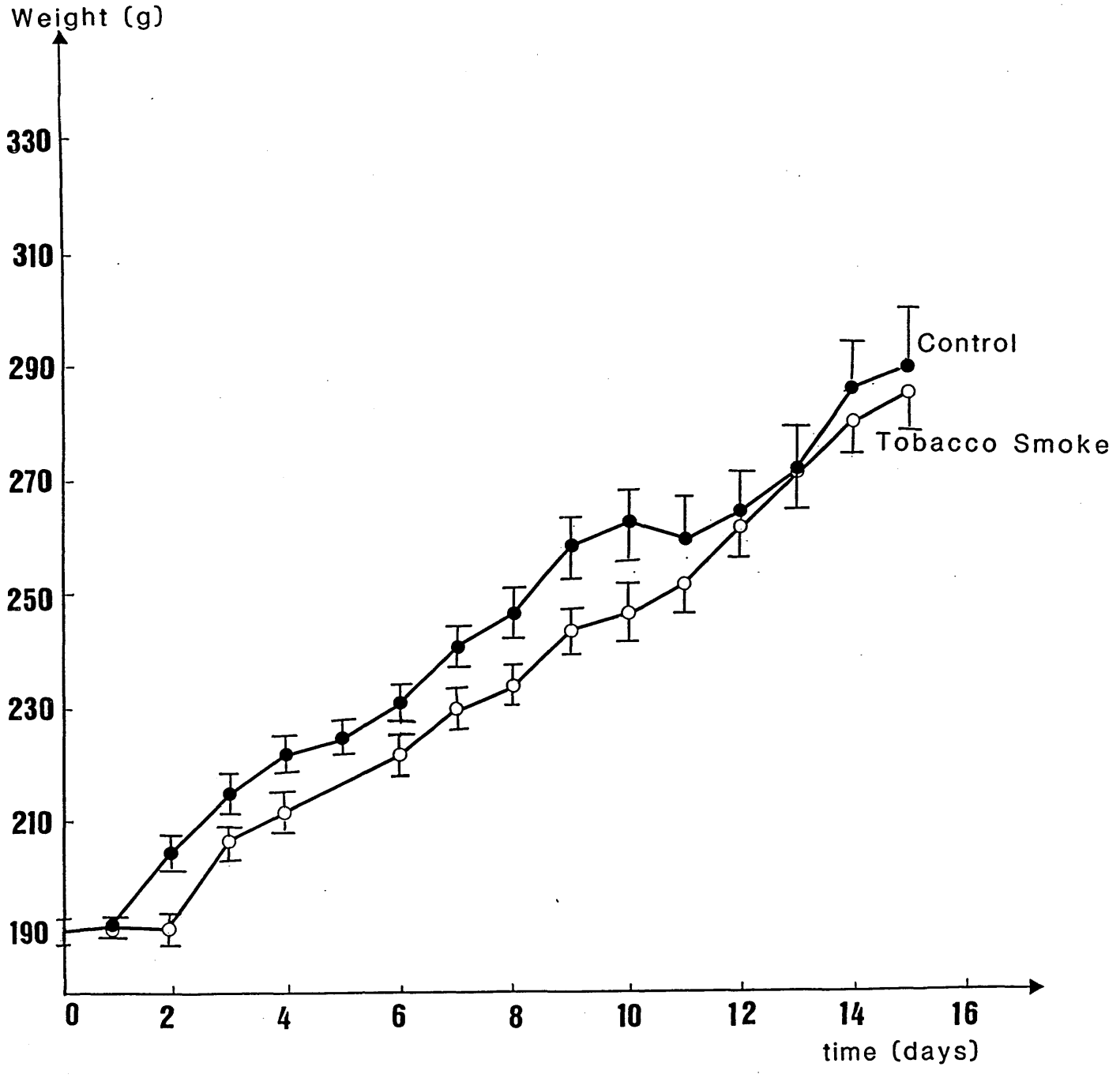
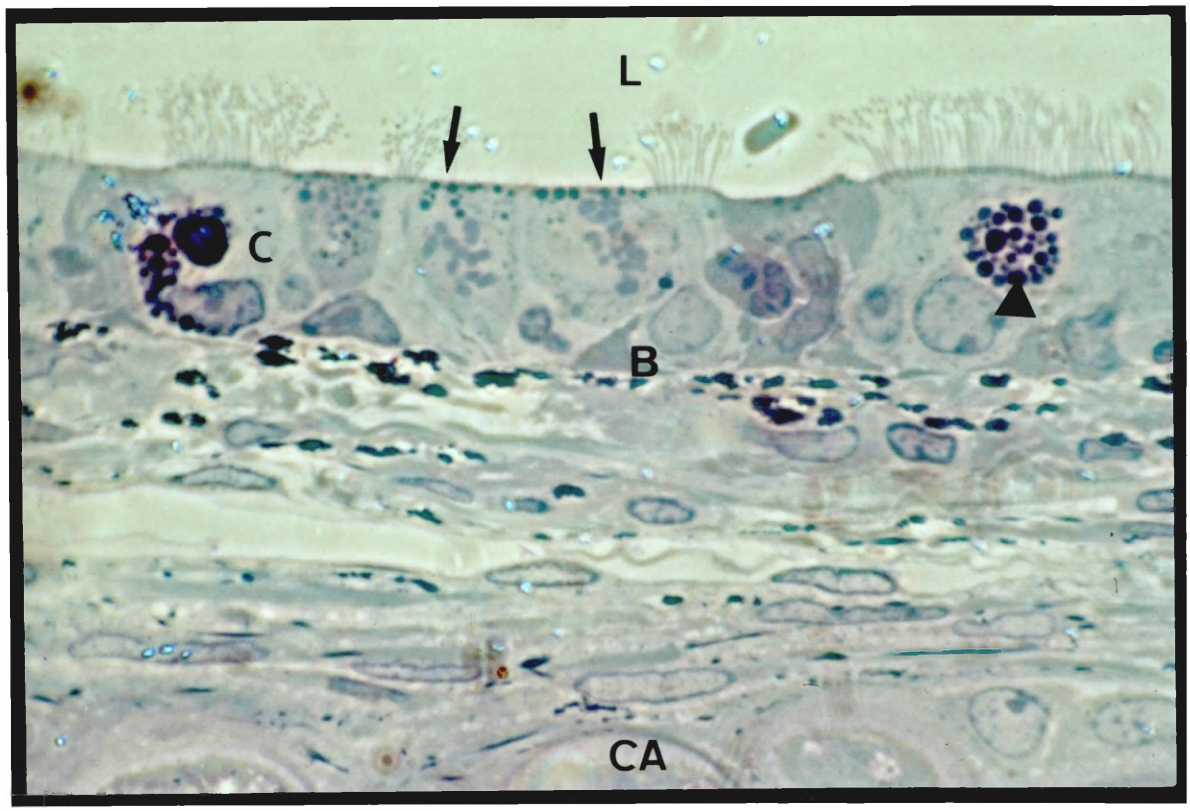
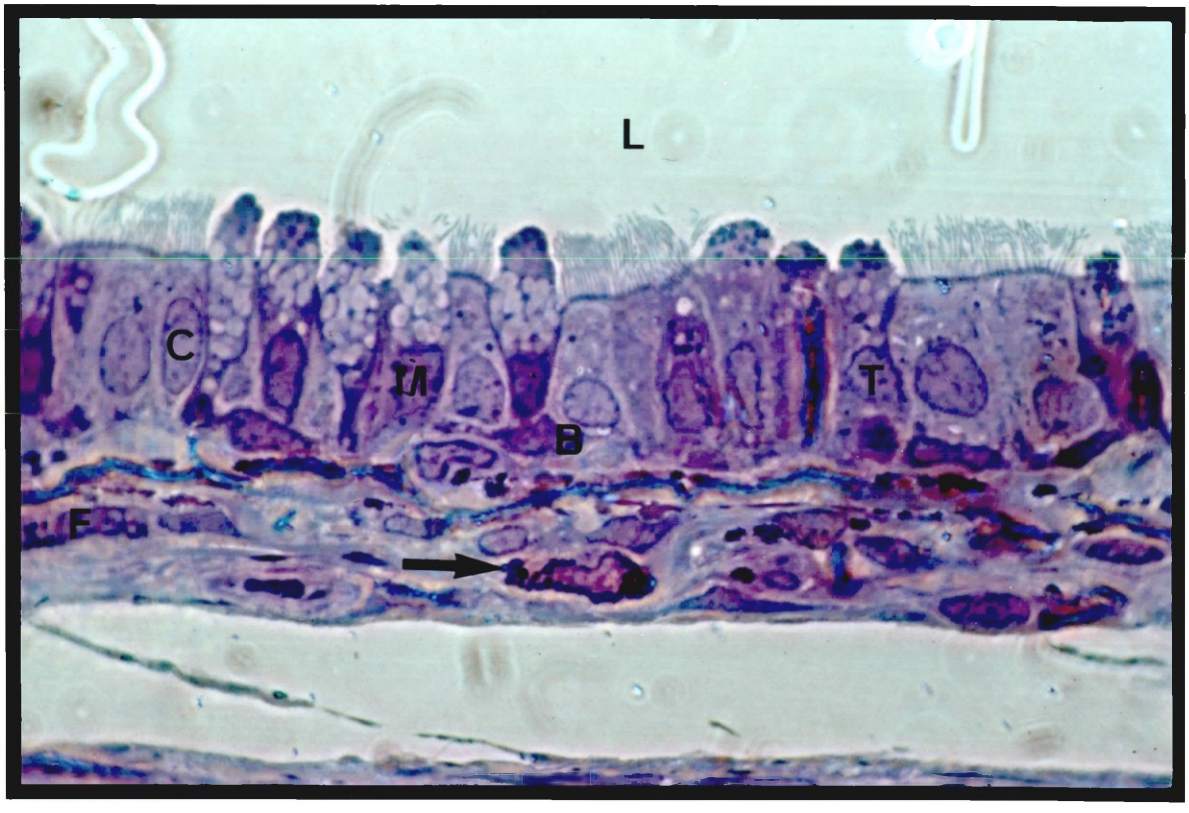


FIGURE 4.2(a) Control epithelium: rat main bronchus.
1μ section stained with Toluidine Blue showing Ciliated cells (C), 2 adjacent Serous cells in mitosis (arrows), Basal cells (B), and intra-epithelial Globular Leukocytes (arrowhead). Lumen (L), Cartilage (CA).
(X 1300, Phase optics)

FIGURE 4.2(b) 14-day TS-exposed epithelium: rat main bronchus.
1μ section stained with Toluidine Blue, showing Ciliated cells (C), which are taller and thinner than their control counterparts. Secretory cells, both Mucous (M) and Transitional (T), contain large volumes of material and protrude above the lumenal surface. Basal cells (B), Fibroblasts (F), Sub-epithelial mast cell (arrow), Lumen (L).
(X 1300, Phase optics)


L
C
B
CA


L
C
M
T
B
F

unit length epithelium were counted on days 0, 1, 3, 7, 10 and 14 and changes in both control and TS-exposed groups were analysed (see Chapter 2, Section 6) (Figs 4.3 - 4.9, Tables 4.1 & 4.2).

Fig 4.3 (a&b), shows that in both sets of experiments, **total cell numbers** were significantly greater in TS-exposed animals than control animals after 14 days.

Changes induced by TS in the mean cell concentration (number/unit distance) and proportions of **each cell type** in both experiments are displayed in Figs 4.3 - 4.9 and Tables 4.1 and 4.2 Comparisons between values for the TS-exposed group at 14 days and day 0 and day 14 control values were made. The day 14 comparisons showed that the numbers of ciliated, mucous, transitional and basal cells were increased significantly and serous cell numbers were significantly decreased after TS-exposure in both sets of experiments. The same comparison for indeterminate cells showed no difference in one set of experiments (2&4), but a TS-induced significant decrease in the other (1&3). Although changes in the numbers of each cell type occurred in control as well as the TS-exposed groups, the latter showed the most pronounced changes. This difference was more prominent in Experiments 1&3, giving a clearer contrast between control and TS-exposed animals eg. mucous cells clearly increased ($p < 0.001$) in response to TS in Experiments 1&3, but showed a much smaller, though significant increase ($p < 0.01$) in Experiments 2&4 (Fig. 4.6 a&b). Table 4.3 summarizes significant changes in numbers of airway epithelial cells induced by 14 days of TS exposure, compared with changes observed in control groups over the same time.

FIGURE 4.3 TOTAL EPITHELIAL CELLS/10 HPF (± SEM) VS TIME

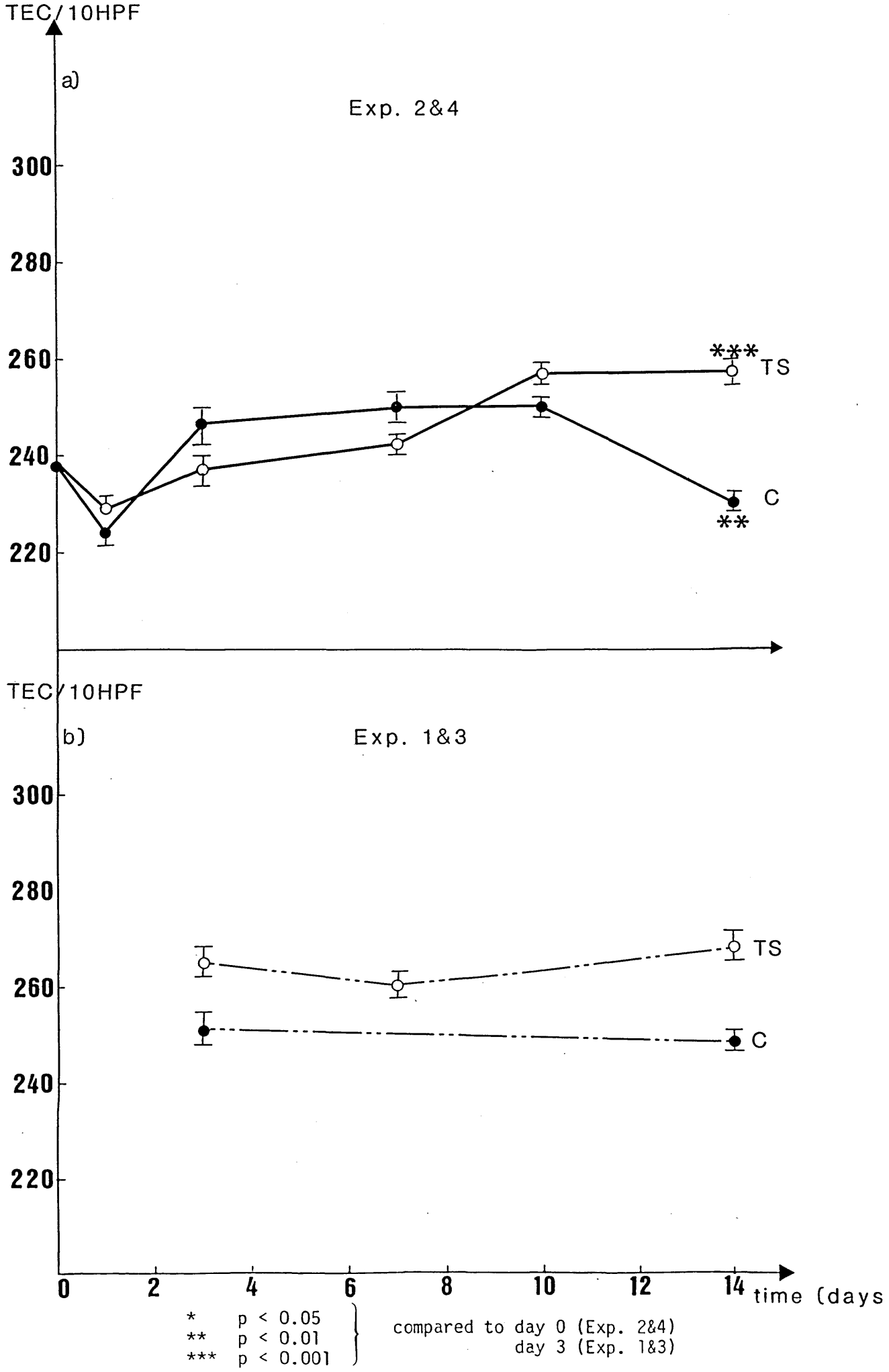


FIGURE 4.4 CILIATED CELLS/10 HPF (± SEM) VS TIME

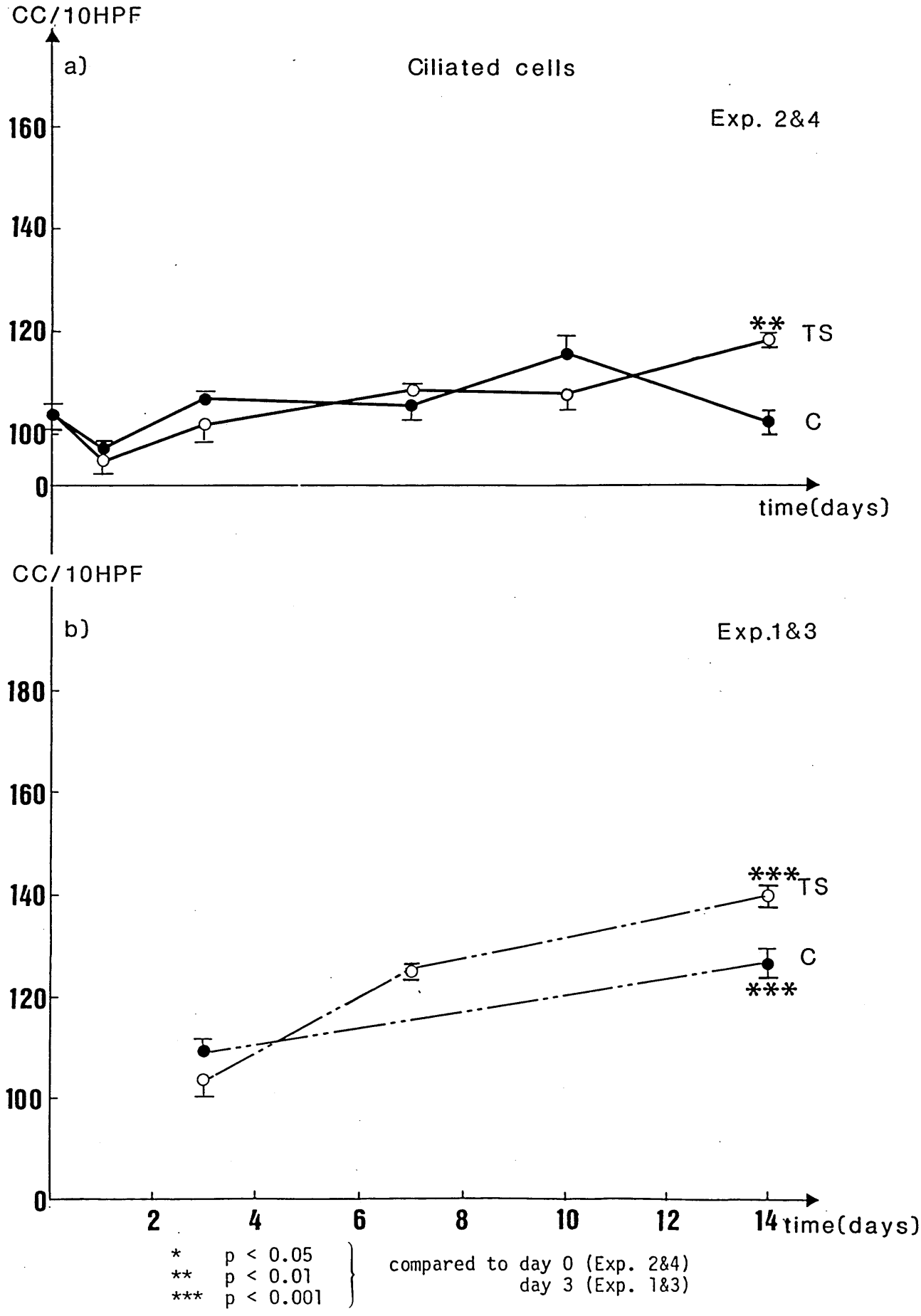


FIGURE 4.5 SEROUS CELLS/10 HPF (± SEM) VS TIME

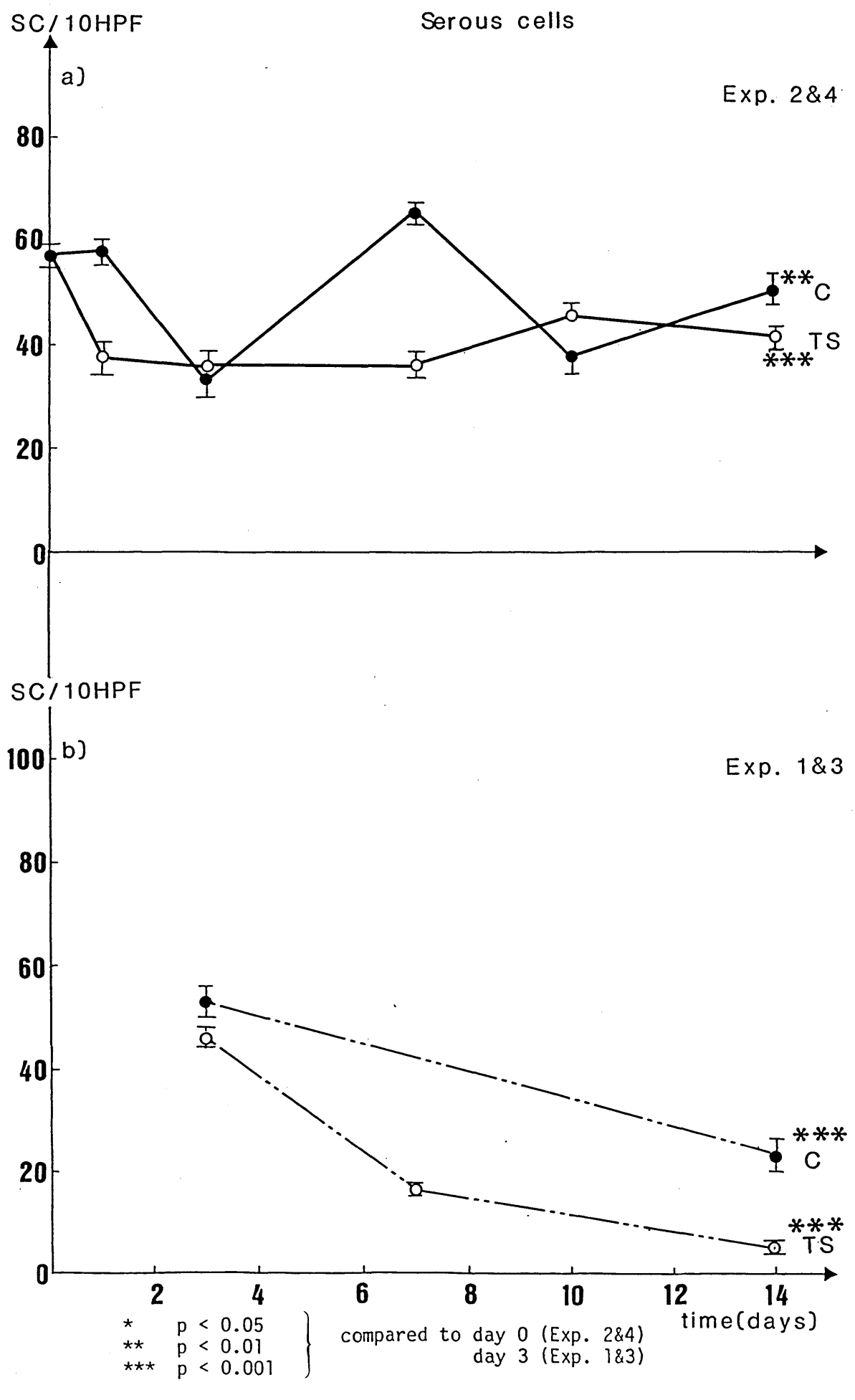


FIGURE 4.6 MUCOUS CELLS/10 HPF (± SEM) VS TIME

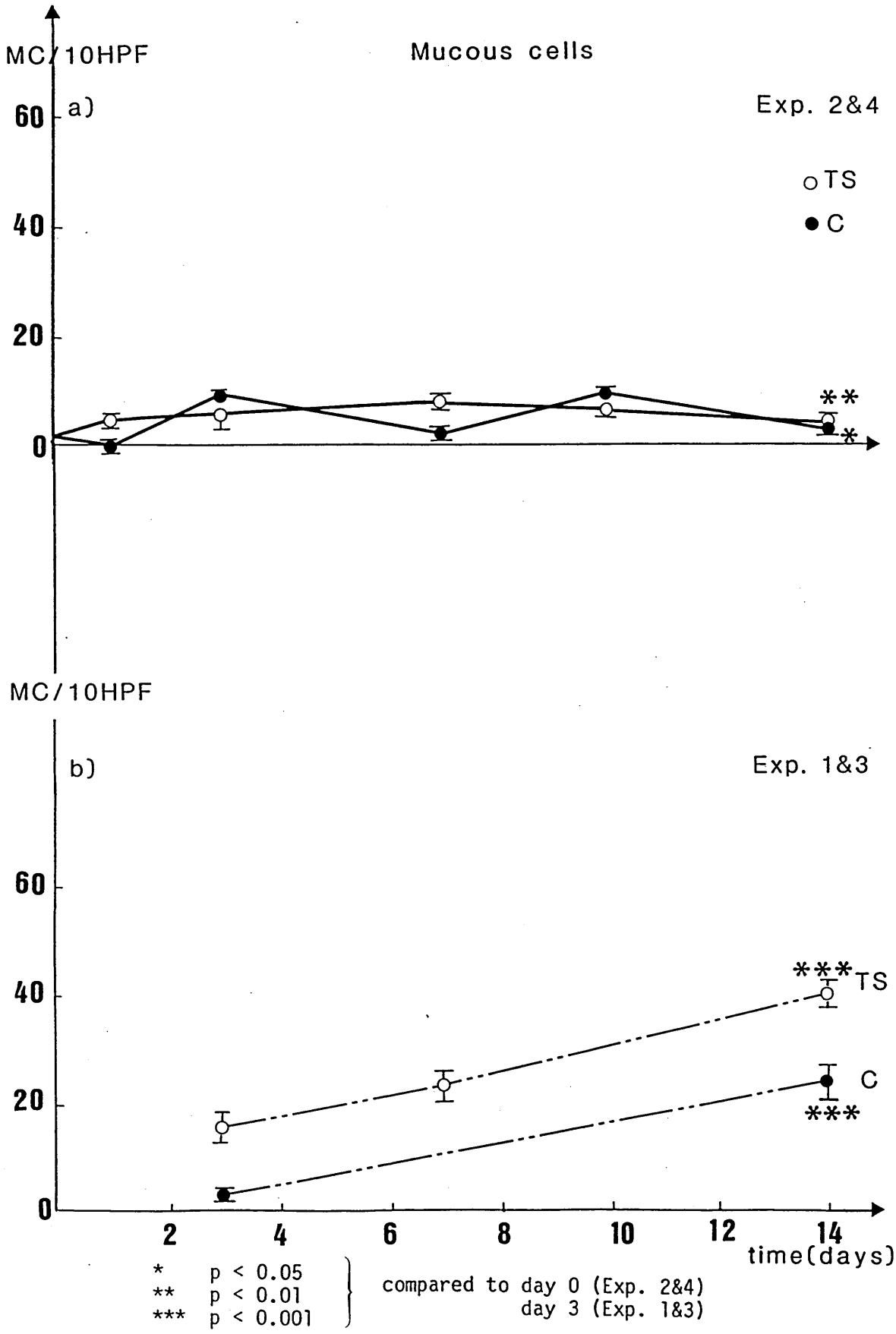


FIGURE 4.7 TRANSITIONAL CELLS/10 HPF (+ SEM) VS TIME

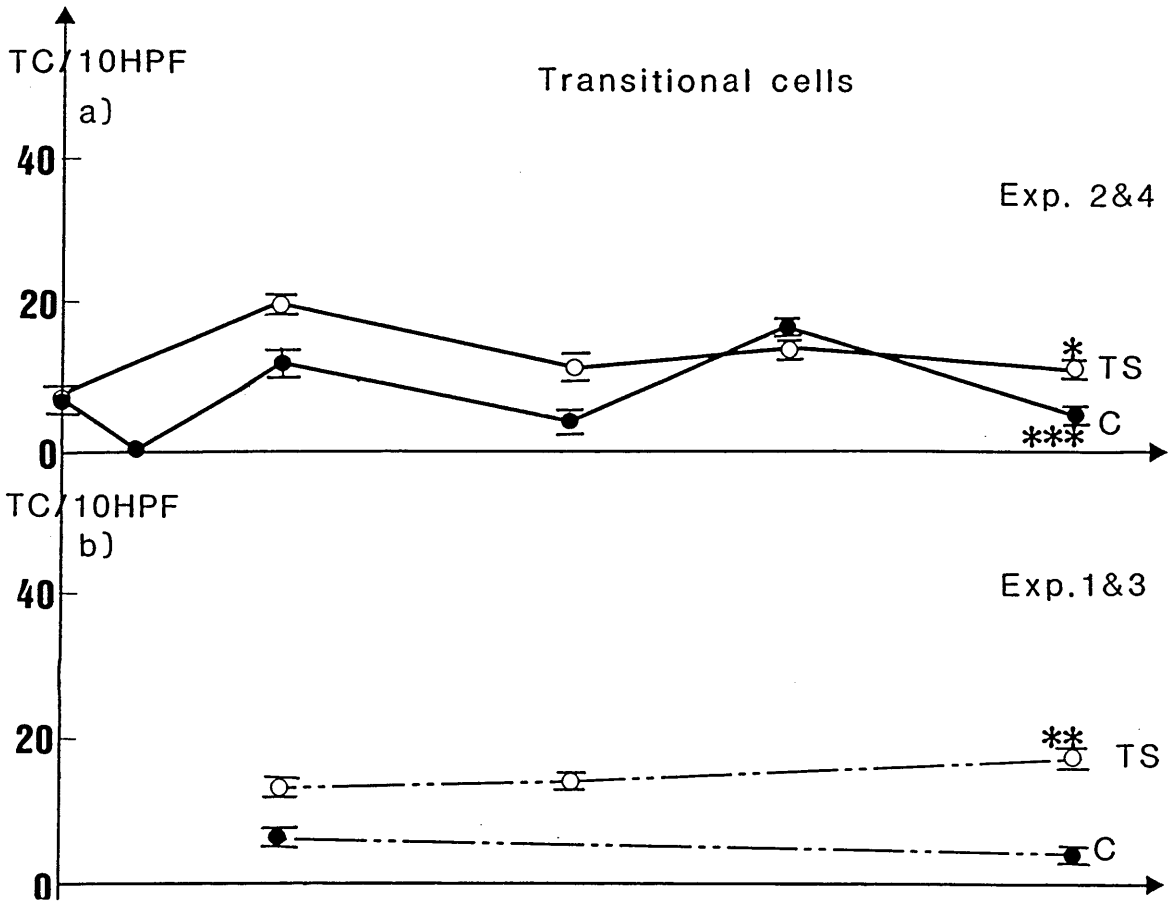
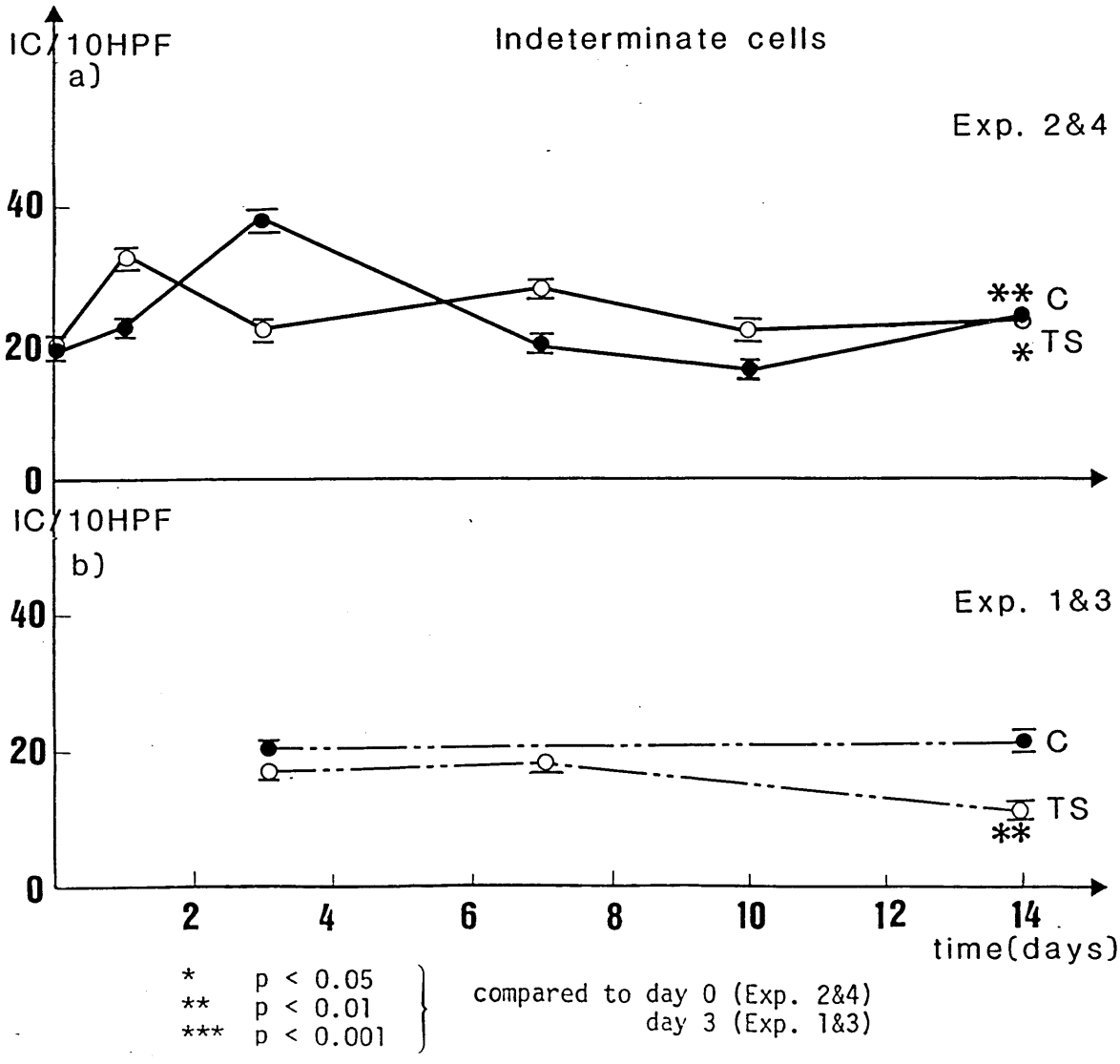


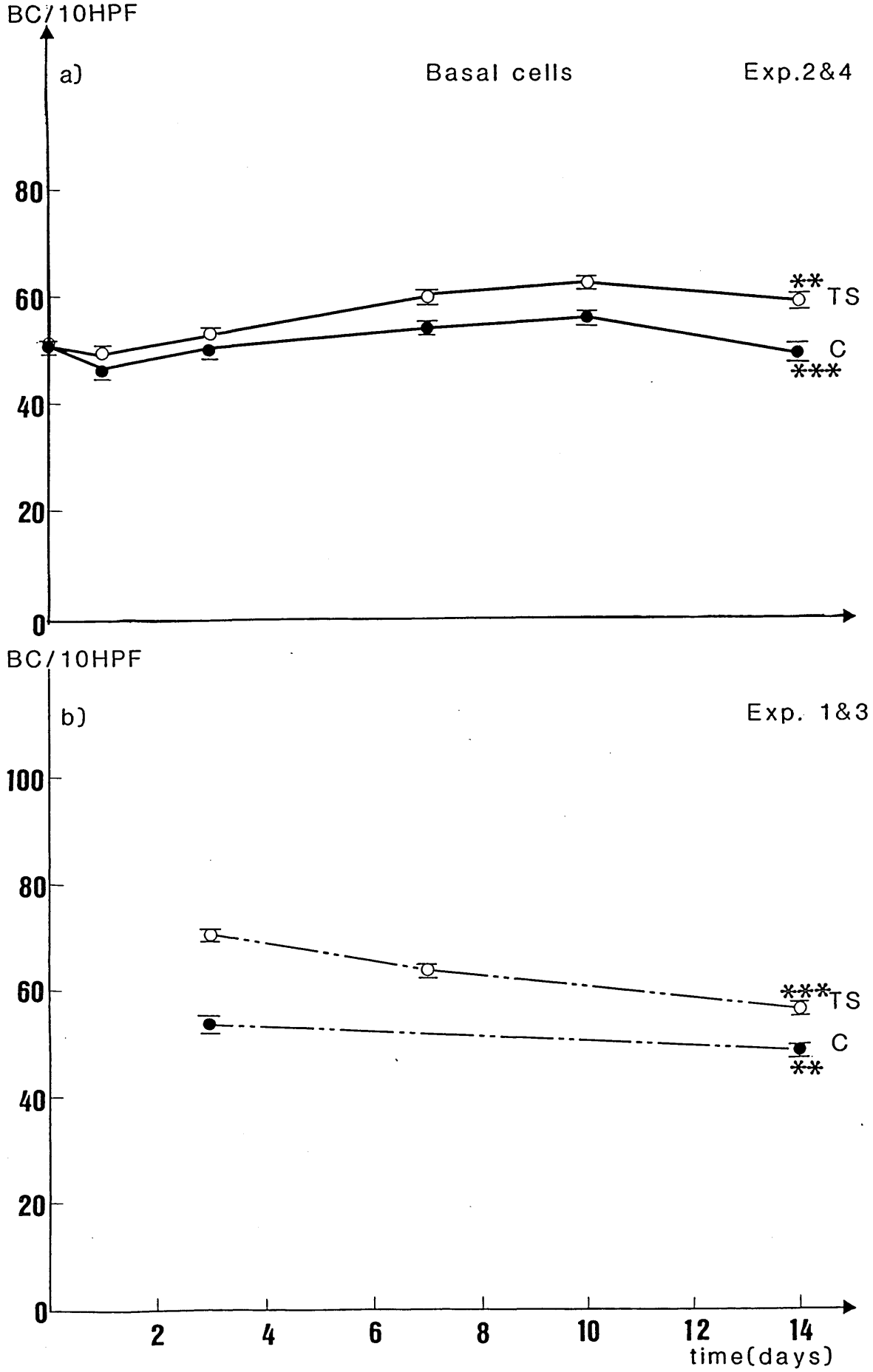
FIGURE 4.8 INDETERMINATE CELLS/10 HPF (+ SEM) VS TIME



* p < 0.05
** p < 0.01
*** p < 0.001

compared to day 0 (Exp. 2&4)
day 3 (Exp. 1&3)

FIGURE 4.9 BASAL CELLS/10 HPF (± SEM) VS TIME



* p < 0.05
** p < 0.01
*** p < 0.001

compared to day 0 (Exp. 2&4)
day 3 (Exp. 1&3)

TABLE 4.1

TOBACCO SMOKE

Epithelial Cell Numbers/10 HPF (± SEM)

CELL TYPE	Time (days) 0	1	3	7	10	14
CILIATED						
Exp 2&4	103.4 (±1.8)	93.8 (±1.7)	101.1 (±2.1)	107.8 (±1.5)	107.1 (±1.7)	117.9 (±1.7)**
Exp 1&3			103.1 (±2.1)	125.2 (±1.9)		139.6 (±1.6)***
SEROUS						
Exp 2&4	56.9 (±1.4)	37.5 (±1.7)	35.8 (±1.7)	35.7 (±2.2)	45.1 (±1.6)	41.3 (±1.5)***
Exp 1&3			45.9 (±2.2)	16.0 (±1.5)		5.0 (±0.6)***
MUCOUS						
Exp 2&4	0.7 (±0.15)	4.1 (±0.6)	4.7 (±0.8)	7.1 (±1.2)	6.4 (±0.7)	3.0 (±0.05)**
Exp 1&3			15.3 (±1.9)	22.8 (±2.0)		38.9 (±2.1)***
TRANS-ITIONAL						
Exp 2&4	7.2 (±0.2)	11.8 (±0.5)	20.3 (±0.8)	10.9 (±0.7)	14.0 (±0.6)	13.3 (±0.6)*
Exp 1&3			12.7 (±1.1)	14.4 (±0.9)		17.3 (±1.1)**
INDETER-MINATE						
Exp 2&4	18.9 (±0.7)	32.4 (±1.4)	21.9 (±0.8)	27.7 (±1.0)	21.7 (±0.8)	22.5 (±0.9)*
Exp 1&3			17.2 (±1.2)	18.6 (±0.9)		10.9 (±0.5)**
BASAL						
Exp 2&4	51.4 (±0.6)	49.7 (±0.7)	52.9 (±1.0)	59.7 (±1.0)	62.5 (±0.6)	59.1 (±0.8)**
Exp 1&3			70.5 (±1.3)	63.9 (±0.9)		56.6 (±0.7***
TOTAL						
Exp 2&4	238.4 (±1.9)	228.7 (±1.9)	236.7 (±2.2)	242.0 (±2.1)	257.0 (±1.7)	257.1 (±2.2)**
Exp 1&3			265.0 (±2.9)	259.9 (±2.4)		268.1 (±3.6) NS

NS Not significantly different
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

} compared to day 0, Exp. 2 & 4
compared to day 3, Exp. 1 & 3

TABLE 4.2

TOBACCO SMOKE

% Epithelial Cell Type (± SEM)

CELL TYPE	Time (days) 0	1	3	7	10	14
CILIATED						
Exp 2&4	43.2 (±0.5)	40.8 (±0.5)	42.6 (±0.7)	44.5 (±0.5)	41.5 (±0.5)	45.8 (±0.5)*
Exp 1&3			39.1 (±0.7)	48.3 (±0.7)		52.3 (±0.5)***
SEROUS						
Exp 2&4	24.0 (±0.6)	16.6 (±0.8)	15.3 (±0.7)	14.7 (±0.9)	17.9 (±0.7)	14.6 (±0.6)***
Exp 1&3			17.3 (±0.8)	6.1 (±0.6)		1.9 (±0.4)***
MUCOUS						
Exp 2&4	0.3 (±0.06)	1.8 (±0.2)	1.9 (±0.3)	2.9 (±0.5)	2.4 (±0.3)	1.2 (±0.2)**
Exp 1&3			5.6 (±0.7)	8.9 (±0.8)		14.4 (±0.7)***
TRANS-ITIONAL						
Exp 2&4	3.1 (±0.1)	5.1 (±0.2)	8.5 (±0.3)	4.7 (±0.3)	5.3 (±0.2)	5.2 (±0.2)*
Exp 1&3			4.8 (±0.4)	5.5 (±0.3)		6.3 (±0.4)*
INDETER-MINATE						
Exp 2&4	7.9 (±0.3)	14.3 (±0.6)	9.3 (±0.3)	11.5 (±0.5)	8.6 (±0.3)	8.9 (±0.4) NS
Exp 1&3			6.5 (±0.5)	7.1 (±0.3)		4.1 (±0.2)**
BASAL						
Exp 2&4	21.6 (±0.2)	21.8 (±0.3)	22.4 (±0.4)	24.6 (±0.3)	24.4 (±0.2)	23.0 (±0.2)*
Exp 1&3			26.6 (±0.4)	23.9 (±0.6)		21.2 (±0.2)**

NS Not significantly different
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

compared to day 0, Exp. 2 & 4
compared to day 3, Exp. 1 & 3

TABLE 4.3

SIGNIFICANT CHANGES IN:

Number Epithelial Cells/10 HPF

due to 14 days TS

EXP. NO.	TREAT-MENT	CELL TYPE: CILIATED	SEROUS	MUCOUS	TRANSITIONAL	INDETERMINATE	BASAL	TOTAL
	Compared with Control day 14:							
2 &	TS	↑p<0.001	↓p<0.01	↑p<0.05	↑p<0.01	NS	↑p<0.01	↑p<0.001
	Compared with Control day 0:							
4	C	NS	↓p<0.01	↑p<0.05	↓p<0.001	↑p<0.001	↑p<0.05	↓p<0.01
	Compared with Control day 14:							
1 &	TS	↑p<0.001	↓p<0.001	↑p<0.01	↑p<0.001	↓p<0.01	↑p<0.01	↑p<0.001
	Compared with Control day 3:							
3	C	↑p<0.001	↓p<0.001	↑p<0.001	NS	NS	↓p<0.01	NS

NS = Not Significant.

4.4. PROLIFERATION

4.4.1. Total Number of Labelled Cells

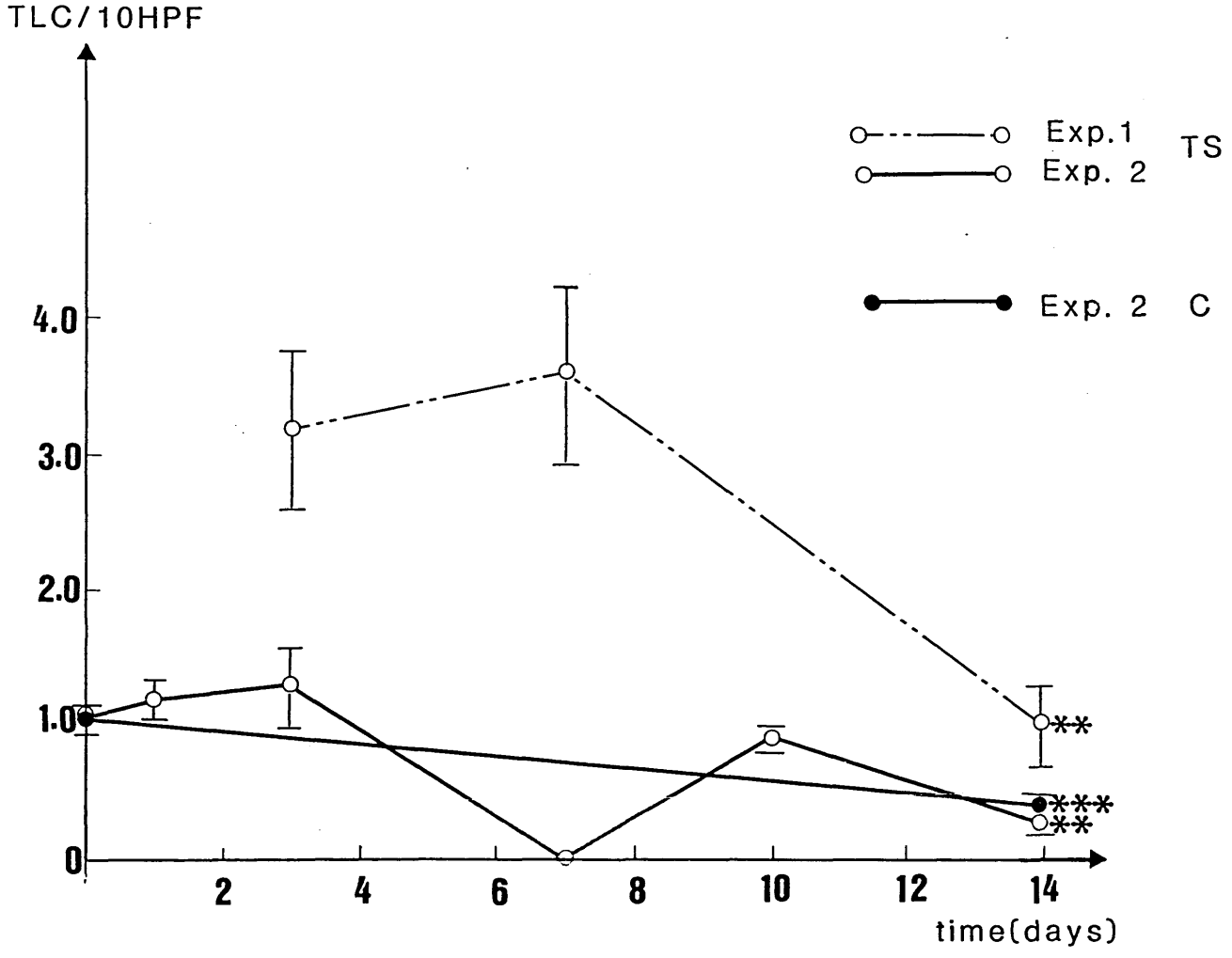
Data from counts of labelled cells in control and TS-exposed animal groups from Experiments 2 and 1 are presented as the mean number of cells labelled per 10 HPF (Figs 4.10, 4.11, Table 4.4); comparisons have been made between values from the day 14 animals exposed to TS and the control values at the beginning and end of the experiments. Fig 4.10 shows that in both experiments after 14 days of TS exposure, the only significant change in the total number of labelled cells is a decrease to below the starting levels ($p<0.01$). There is a notable difference between levels of labelling in the 2 experiments from days 3 to 14.

4.4.2. Number of Each Cell Type Labelled

Fig 4.11 (a-f) shows changes in the mean numbers of each labelled cell type with time of TS exposure in Experiments 2 and 1, and changes in control animals from Experiment 2. There are marked differences between Experiments 2 and 1 in the ciliated, mucous, transitional and basal cell compartments.

In Experiment 2, there was no significant change in labelled ciliated, serous or mucous cell numbers in response to TS. The mean number of labelled transitional cells increased significantly after 3 days of exposure ($p<0.01$) then decreased to a consistently low level

FIGURE 4.10 TOBACCO SMOKE: PROLIFERATION
TOTAL LABELLED EPITHELIAL CELLS/10 HPF (± SEM) VS TIME



* p < 0.05
** p < 0.01
*** p < 0.001

} compared to day 0 (Exp. 2)
day 3 (Exp. 1)

FIGURE 4.11 TOBACCO SMOKE: PROLIFERATION
LABELLED EPITHELIAL CELLS/10 HPF (± SEM) VS TIME

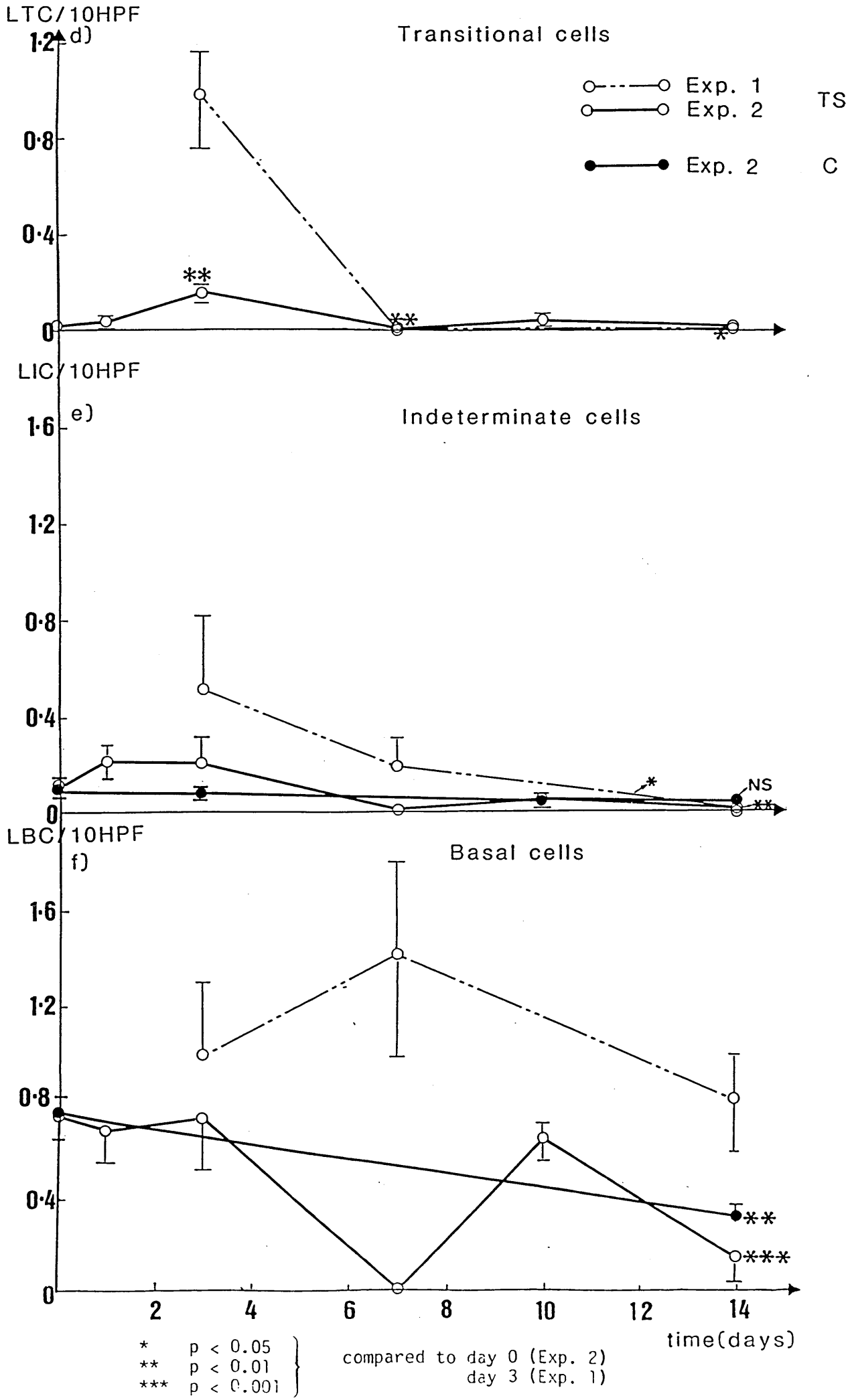


TABLE 4.4 a)

TOBACCO SMOKE - PROLIFERATION - EXPERIMENTS 2 & 1

Labelled Epithelial Cells/10 HPF (± SEM)

CELL TYPE	Time (days) 0	1	3	7	10	14
CILIATED						
Exp 2	0.016 (±0.01)	0	0	0	0	0 NS
Exp 1			0.04 (±0.04)	0.24 (±0.14)		0.04(±0.04)NS
SEROUS						
Exp 2	0.18 (±0.04)	0.26 (±0.07)	0.25 (±0.1)	0	0.16 (±0.05)	0.13(±0.06)NS
Exp 1			0.22 (±0.22)	0.39 (±0.19)		0 NS
MUCOUS						
Exp 2	0	0.015 (±0.015)	0	0	0	0
Exp 1			0.39 (±0.2)	1.45 (±0.6)		0.21(±0.09)NS
TRANS-ITIONAL						
Exp 2	0.016 (±0.01)	0.029 (±0.01)	0.15 (±0.02)**	0	0.02 (±0.01)	0 NS
Exp 1			0.97 (±0.2)	0*		0*
INDETER-MINATE						
Exp 2	0.10 (±0.03)	0.21 (±0.07)	0.2 (±0.12)	0	0.05 (±0.02)*	0 ***
Exp 1			0.51 (±0.3)	0.18 (±0.13)		0 *
BASAL						
Exp 2	0.75 (±0.09)	0.68 (±0.12)	0.72 (±0.2)	0	0.64 (±0.09)	0.13(±0.07)***
Exp 1			0.99 (±0.3)	1.4 (±0.4)		0.79(±0.2) NS
TOTAL CELLS						
Exp 2	1.06 (±0.11)	1.2 (±0.16)	1.3 (±0.3)	0	0.88 (±0.1)	0.26(±0.12)***
Exp 1			3.2 (±0.6)	3.6 (±0.7)		1.0 (±0.3)**

NS Not significantly different
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Compared to day 0 - Exp 2
Compared to day 3 - Exp 1

TABLE 4.4 b)

TOBACCO SMOKE - PROLIFERATION - EXPERIMENTS 2 & 1

%Labelled Epithelial Cells (± SEM)

CELL TYPE	Time (days) 0	1	3	7	10	14	
CILIATED							
Exp 2	0.02 (± .01)	0	0	0	0	0	NS
Exp 1			0.04 (± .04)	0.17 (± .1)		0.03 (± .03	NS
SEROUS							
Exp 2	0.33 (± .07)	0.53 (± .2)	0.68 (± .3)	0	0.28 (± .08)	0.3 (± .16)	NS
Exp 1			0.38 (± .4)	1.5 (± .8)		0	NS
MUCOUS							
Exp 2	0	0.26 (± .26)	0	0	0	0	NS
Exp 1			2.0 (± 1.5)	3.1 (± 1.1)		0.34 (± .15)	NS
TRANS-ITIONAL							
Exp 2	0.12 (± .06)	0.2 (± 0.17)	0.81 (± .2)	0	0.27 (± .1)	0	NS
Exp 1			9.8 (± 1.6)	0		0	**
INDETER-MINATE							
Exp 2	0.56 (± .2)	0.68 (± .2)	0.99 (± .7)	0	0.25 (± .1)	0	**
Exp 1			4.0 (± 2.4)	1.0 (± .7)		0	*
BASAL							
Exp 2	1.5 (± .2)	1.3 (± .2)	1.5 (± .4)	0	1.0 (± .1)	0.2 (± .1)	***
Exp 1			1.5 (± .4)	2.3 (± .6)		1.4 (± .4)	NS
TOTAL							
Exp 2	0.45 (± .05)	0.5 (± .07)	0.6 (± .1)	0	0.3 (± .04)	0.09 (± .04)	***
Exp 1			1.2 (± .2)	1.45 (± .3)		0.4 (± .1)	**

NS Not significantly different
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Compared to day 0 - Exp 2
Compared to day 3 - Exp 1

TABLE 4.5

PROLIFERATION - SIGNIFICANT CHANGES IN:

Labelled Epithelial Cells/10 HPF

14 days of TS-exposure compared with { Day 0 (Exp. 2) / Day 3 (Exp. 1)

	CELL TYPE						
EXP. NO.	CILIATED	SEROUS	MUCOUS	TRANSITIONAL	INDETERMINATE	BASAL	TOTAL
Exp 2	NS	NS	NS	↑$p < 0.02$ ↓$p < 0.01$	↓$p < 0.01$	↓$p < 0.001$	↓$p < 0.001$
Exp 1	NS	NS	NS	↓$p < 0.05$	↓$p < 0.05$	NS	↓$p < 0.01$
Control (Exp 2)	NS	↓$p < 0.05$	0	NS	NS	↓$p < 0.01$	↓$p < 0.001$

NS = Not Significant.

by 7 days. Labelled indeterminate and basal cell numbers decreased significantly after 14 days. There were no labelled cells of any type found in the one 7 day TS-exposed animal.

In Experiment 1, after 3 days of TS exposure, all cell compartments (except serous) showed higher numbers of proliferating cells than at the same time in Experiment 2. Although changes were observed in the mean number of labelled cells in all cell compartments (Fig 4.11 a-f), large standard errors rendered them insignificant for all but transitional and indeterminate cells, where there was a significant decrease from the high 3 day level. Table 4.5 summarises significant changes observed in labelled cell numbers during TS exposure.

4.4.3. Percentage Label Distribution

When the labelled cell concentration data for each cell type are expressed as a percentage of the total labelled population (Table 4.6), the relative contribution of each cell type to the overall proliferative response in each experiment can be seen.

In Experiment 2 control (day 0) animals, basal and serous cells were the major contributors to the proliferating population. Between 1 and 10 days of TS exposure, indeterminate, then transitional cells proportionately increased (to 18% and 12%) then decreased (to 6% and 2%) their contribution and by 14 days, the proliferating population was again composed of basal and serous

TABLE 4.6

TOBACCO SMOKE - PROLIFERATION

% Label Distribution

Time (days)	CELL TYPE					
	CILIATED	SEROUS	MUCOUS	TRANSITIONAL	INDETERMINATE	BASAL
			Experiment 2			
0	2	17	0	2	9	71
1	0	22	1	2	18	57
3	0	19	0	12	15	55
10	0	18	0	2	6	73
14	0	50	0	0	0	50
			Experiment 1			
3	1	7	12	30	16	31
7	7	11	40	0	5	39
14	4	0	21	0	0	79

cells, but in equal proportions (Table 4.6).

In Experiment 1, after 3 days of TS exposure, mucous-secreting cells (both transitional and mucous cells) made up 42% of the labelled cell population, indeterminate cells were 16% and basal cells 31%. For the rest of the experiment the contribution from mucous cells remained high, 40% and 21% at 7 days and 14 days respectively, by which time the basal proportion of the labelled population had returned to 79%, close to the control value for the basal proportion in Experiment 2 (Table 4.6).

Comparison of TS exposed and control data shows that whereas in controls, label distribution between basal, serous and indeterminate cells remained relatively constant for 14 days, (see Table 3.4) TS increased the indeterminate and transitional proportion of the labelled population in Experiment 2 and the transitional and mucous cell proportion in Experiment 1.

The biological significance of the changes will be examined in the discussion.

4.5. DIFFERENTIATION

The following data (Figs 4.12 a&b, 4.13 a-f, Tables 4.7&8) represent counts of cells which became labelled after a single administration of ^{3}H-T given as a pulse at the beginning of Experiments 4 and 3. Thereafter, treated animal groups were exposed to TS and tissue samples were taken during exposure at 24 h, 3, 7,

10, and 14 days (Experiment 2) and 3, 7 and 14 days (Experiment 1). The single pulse of ^{3}H-T labelled a cohort of dividing cells; change in label distribution was followed during TS exposure by counting the numbers of all labelled and unlabelled cells at each time point. Comparison was made with control values at the beginning and end of the experiments.

4.5.1. Total Number of Labelled Cells

For both control and TS-exposed animals in Experiment 4, the concentration of total labelled cells decreased with time during the experiment (Fig 4.12 a&b, Tables 4.7); by 14 days, 31% of the original labelled cell population remained in the TS-exposed animals. In Experiment 3, although there were no significant changes between days 3 and 14 in the control animals, labelled cell numbers in the TS-exposed animals decreased significantly ($p < 0.05$) at day 7, then returned to day 3 levels by the end of the experiment.

4.5.2 Number of Each Cell Type Labelled

Fig 4.13 (a-f) and Table 4.7 show the change of label distribution in both TS-exposed and control animal groups with time in Experiment 4. The largest changes are in the basal and ciliated cell compartments. Although the absolute number of labelled ciliated cells was low initially, there was a steady increase, which was significantly different from controls at days 10 and 14 ($p < 0.05$), contrasting with control animals where there was no significant change. The transitional cell compartment also showed a significant increase in its concentration of labelled cells by 14 days. Labelled

FIGURE 4.12 TOBACCO SMOKE: DIFFERENTIATION
TOTAL LABELLED EPITHELIAL CELLS/10 HPF (± SEM)

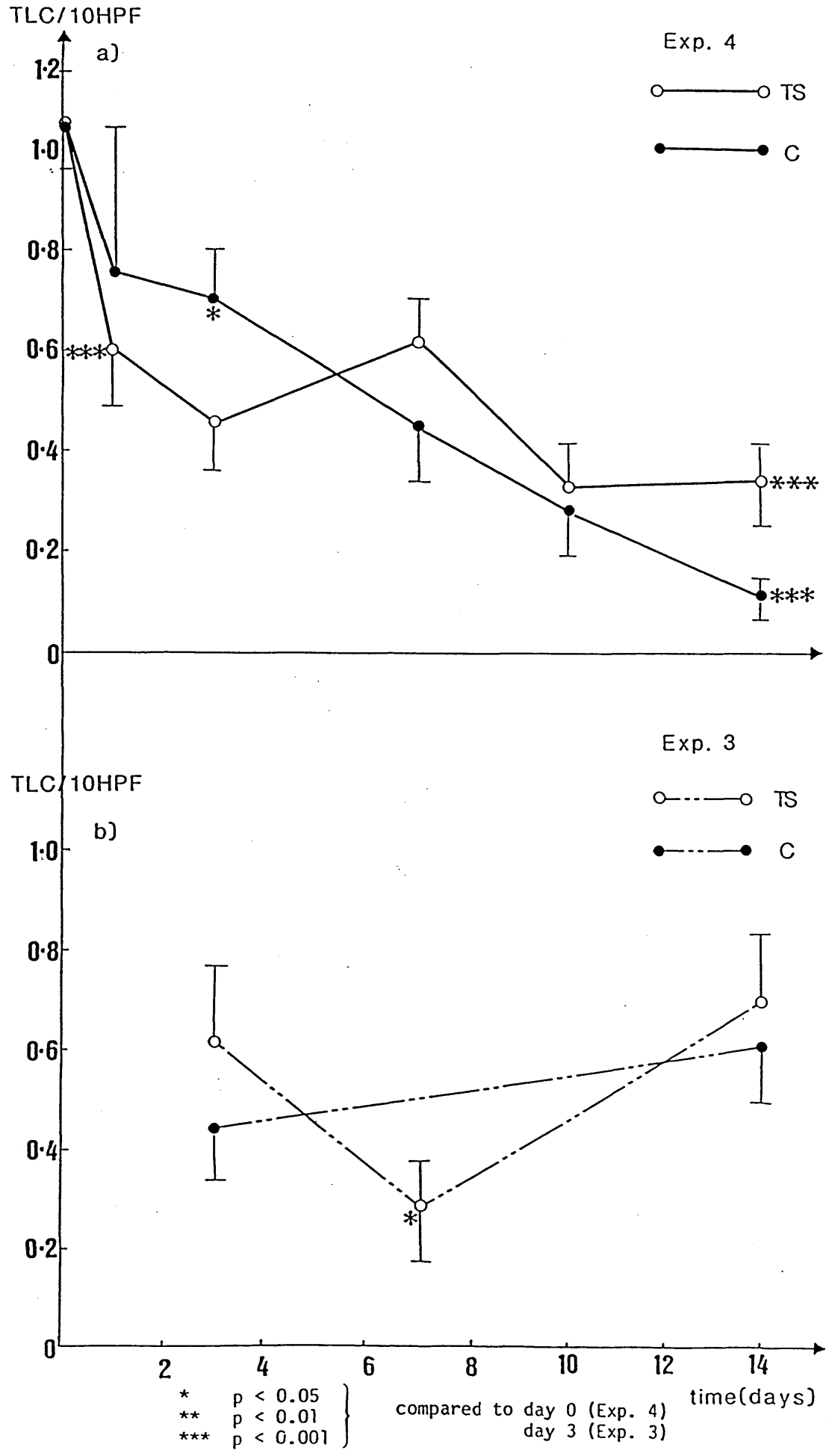


FIGURE 4.13 TOBACCO SMOKE: DIFFERENTIATION
LABELLED EPITHELIAL CELLS/10 HPF (± SEM)

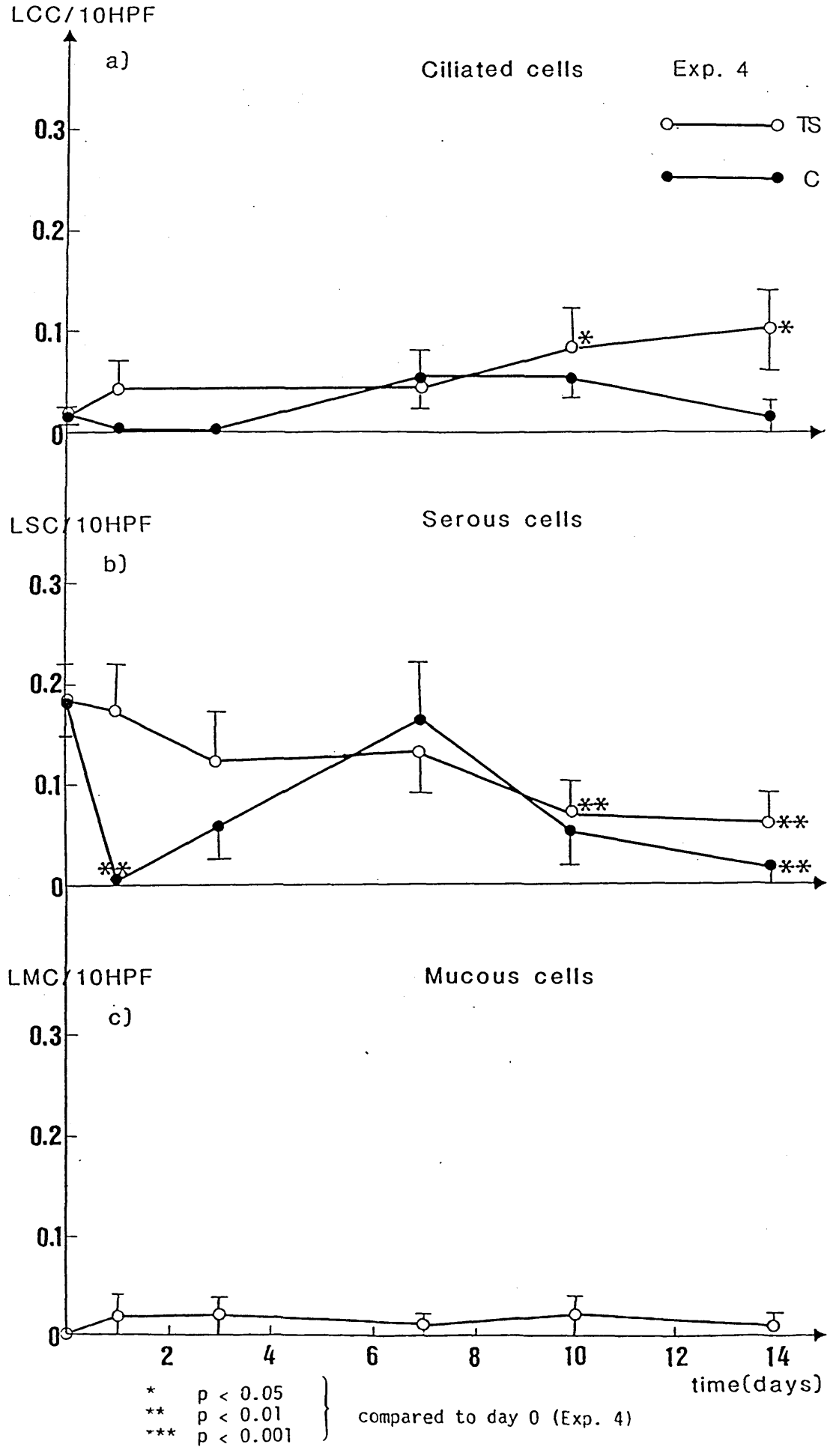


FIGURE 4.13 TOBACCO SMOKE: DIFFERENTIATION
LABELLED EPITHELIAL CELLS/10 HPF (± SEM)

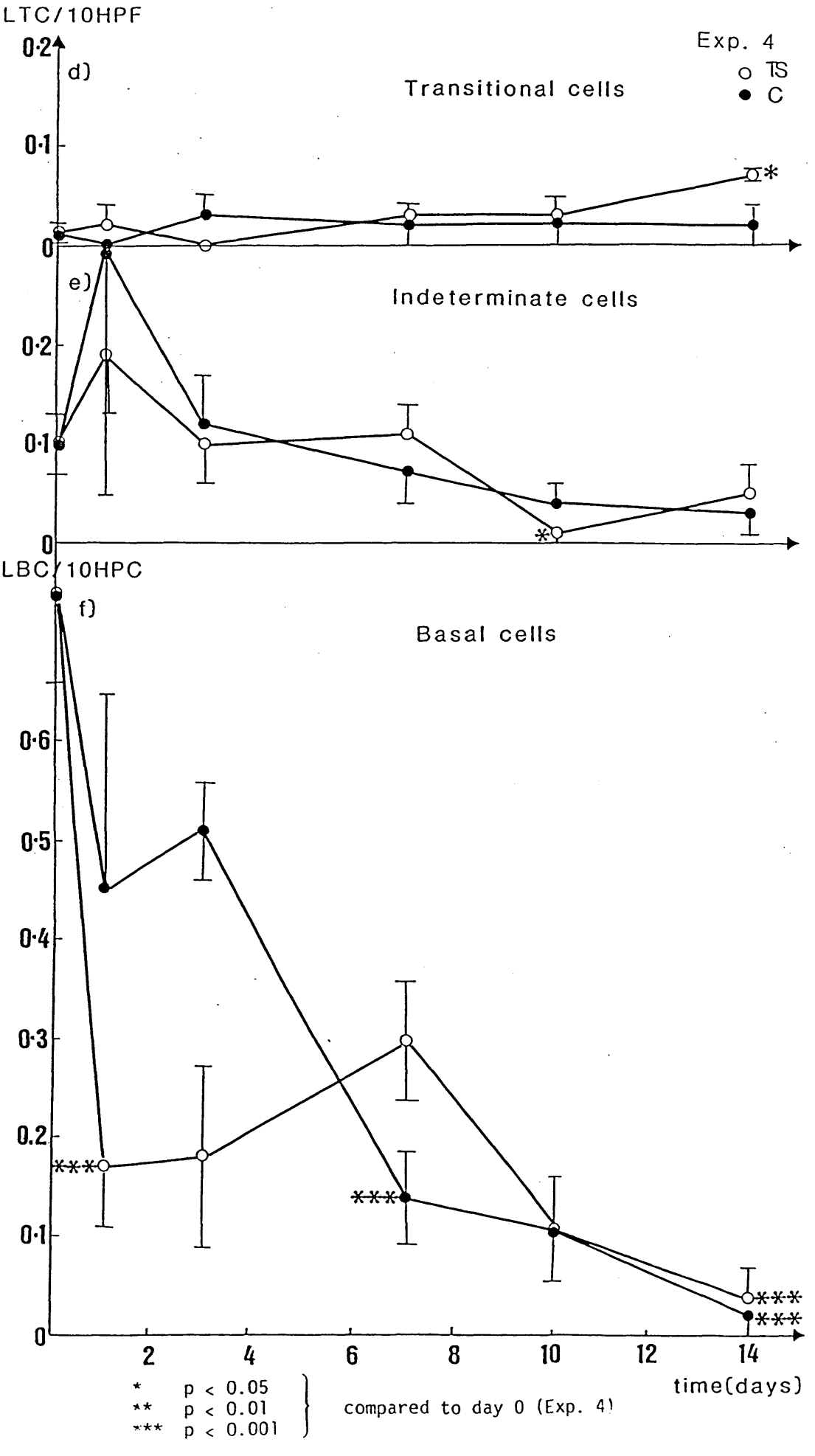


FIGURE 4.14 TOBACCO SMOKE: DIFFERENTIATION
LABELLED EPITHELIAL CELLS/10 HPF (± SEM)

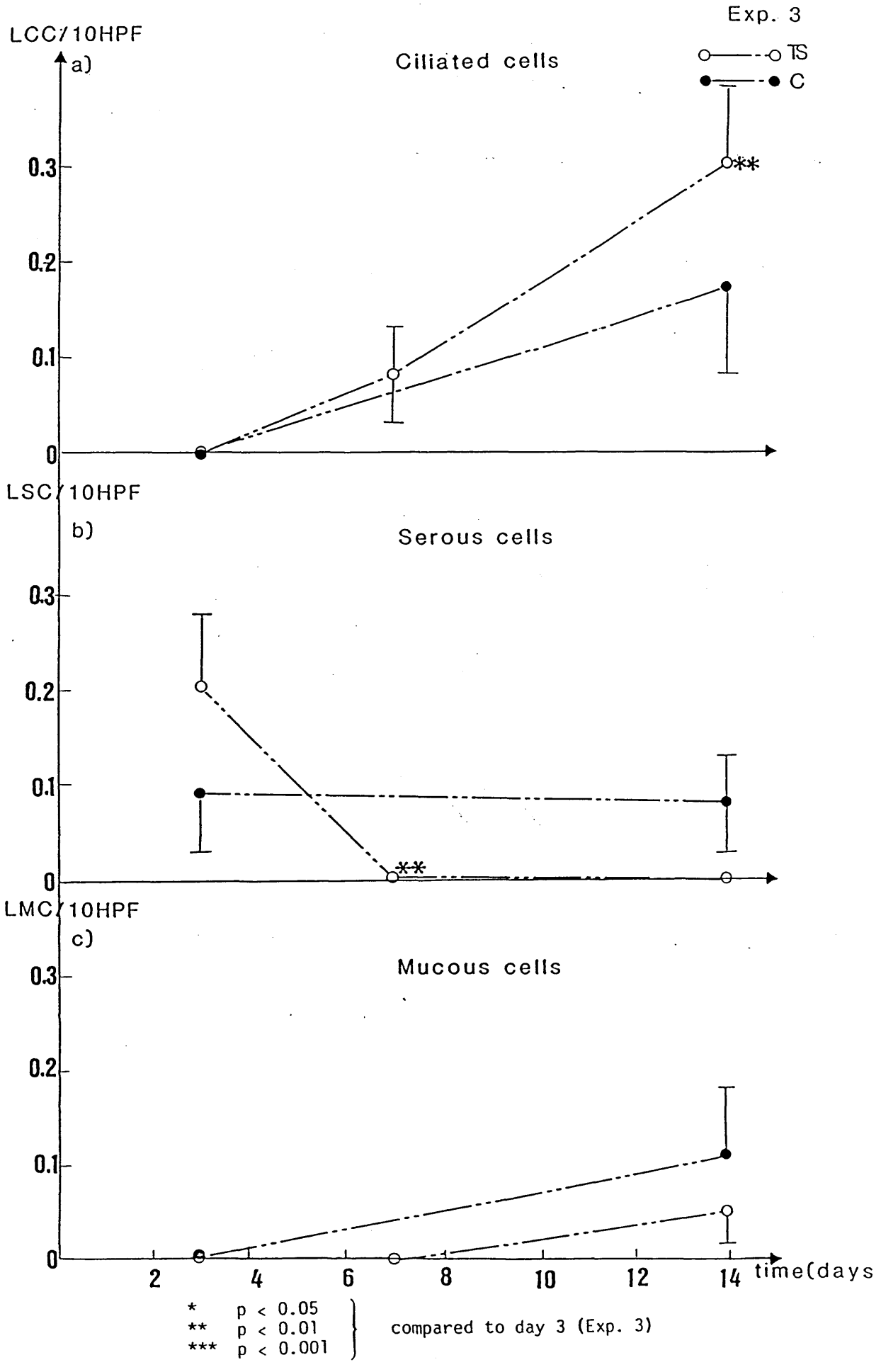
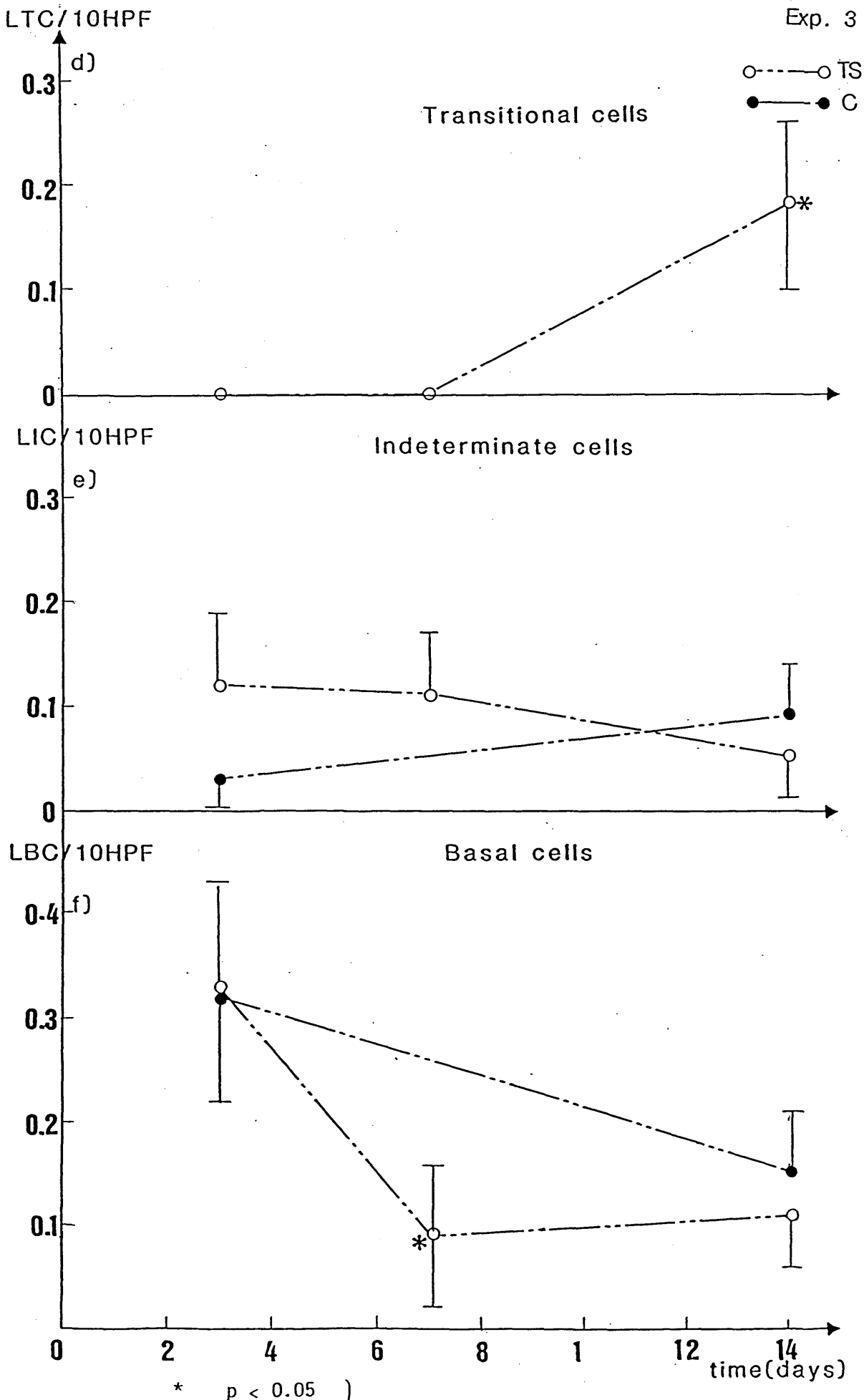


FIGURE 4.14 TOBACCO SMOKE: DIFFERENTIATION
LABELLED EPITHELIAL CELLS/10 HPF (± SEM)



* p < 0.05
** p < 0.01
*** p < 0.001

compared to day 3 (Exp. 3)

TABLE 4.7

TOBACCO SMOKE - DIFFERENTIATION - EXPERIMENTS 4 & 3

Labelled Epithelial Cells/10 HPF (± SEM)

CELL TYPE	Time (days) 0	1	3	7	10	14
CILIATED						
Exp 4	0.016 (±0.01)	0.04 (±0.03)	0.04 (±0.04)	0.04 (±0.02)	0.08 (±0.04)*	0.1 (±0.04)*
Exp 3			0	0.08 (±0.05)		0.3 (± 0.08)**
SEROUS						
Exp 4	0.18 (±0.04)	0.17 (±0.05)	0.12 (±0.05)	0.13 (±.04)	0.07 (±0.03)*	0.06 (±0.03)**
Exp 3			0.2 (±0.08)	0		0 **
MUCOUS						
Exp 4	0	0.02 (±0.02)	0.02 (±0.02)	0.01 (±0.01)	0.02 (±0.02)	0.01 (±0.01) NS
Exp 3			0	0		0.05 (±0.03) NS
TRANS-ITIONAL						
Exp 4	0.016(±0.01)	0.02(±0.02)	0	0.03 (±0.01)	0.03 (±0.02)	0.07 (±0.004) *
Exp 3			0	0		0.18 (±0.08) *
INDETER-MINATE						
Exp 4	0.10 (±0.03)	0.19 (±0.06)	0.10 (±0.04)	0.11 (±0.03)	0.01 (±0.01)*	0.05 (±0.02) NS
Exp 3			0.12 (±0.07)	0.11 (±0.06)		0.05 (±0.04) NS
BASAL						
Exp 4	0.75 (±0.09)	0.17 (±0.06)***	0.18 (±0.06)	0.3 (±0.06)	0.11 (±0.05)	0.04 (±0.03)***
Exp 3			0.33 (±0.1)	0.1 (±0.07)		0.11 (±0.05) NS
TOTAL CELLS						
Exp 4	1.06 (±0.11)	0.60 (±0.11)	0.45 (±0.09)	0.61 (±0.09)	0.32 (±0.09)	0.33 (±0.08)***
Exp 3			0.61 (±0.16)	0.3 (±0.1)*		0.7 (±0.14) NS

NS Not significantly different
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Compared to day 0 - Exp 4
Compared to day 3 - Exp 3

TABLE 4.8

DIFFERENTIATION - SIGNIFICANT CHANGES IN:

Labelled Epithelial Cells/10 HPF

14 days of TS-exposure compared with { Day 0 (Exp. 4) / Day 3 (Exp. 3)

EXP. NO.	TREAT-MENT	CELL TYPE: CILIATED	SEROUS	MUCOUS	TRANSITIONAL	INDETERMINATE	BASAL	TOTAL
4	TS	↑p<0.05	↓p<0.01)	NS	↑p<0.05	↓p<0.05	↓p<0.001	↓p<0.01
	C	NS	↓p<0.01	0	NS	NS	↓p<0.001	↓p<0.001
3	TS	↑p<0.01	↓p<0.01	NS	↑p<0.05	NS	↓p<0.05	↓p<0.05 ↑p<0.05
	C	NS	NS	NS	0	NS	NS	NS

NS = Not Significant.

basal cell numbers in the TS-exposed epithelium fell sharply and significantly ($p<0.001$) by 24 h and decreased again between 7 and 14 days. There was also a large decrease in control labelled basal cell concentration but it did not reach significance until 7 days. Labelled serous and indeterminate cells showed significant decreases by 10 days and there were no significant changes in the mucous cell compartment.

In Fig 4.14 (a-f) and Table 4.7, the changes in label distribution between cell compartments in control and TS-exposed groups from Experiment 3 are shown. As in Experiment 4, the concentration of labelled cells increased significantly in the ciliated and transtitional compartments and decreased in the serous and basal compartments. By contrast, in this experiment mucous cells were observed at 14 days and indeterminate cells did not change significantly. Table 4.8 summarizes these changes and compares the findings in the two experiments.

4.5.3 Percentage Label Distribution

When, using the above data, the number of each cell type labelled is expressed as a percentage of all labelled cells (Table 4.9), the shift in distribution of label between cell compartments can be appreciated.

In both experiments, the contribution of basal cells decreased and that of ciliated cells increased throughout the experimental period. Also in both experiments: the mucous-secretory proportion of the labelled population (mucous and transitional cells), increased

TABLE 4.9

TOBACCO SMOKE - DIFFERENTIATION

% Label Distribution

Time (days)	CELL TYPE					
	CILIATED	SEROUS	MUCOUS	TRANSITIONAL	INDETERMINATE	BASAL
			Experiment 4			
0	2	17	0	2	9	71
1	7	28	3	3	32	28
3	9	27	4	0	22	40
7	7	21	2	5	18	49
10	25	22	6	9	3	34
14	30	18	3	21	15	12
			Experiment 3			
3	0	33	0	0	20	54
7	29	0	0	0	37	33
14	43	0	7	26	7	16

steadily; and the contribution of indeterminate cells increased during the first week of exposure and decreased thereafter. In Experiment 4 the proportion of labelled serous cells changed little with time, while in Experiment 3 there were no labelled serous cells after 3 days.

When these data (Table 4.9) were compared to corresponding control figures (see Table 3.8), a similar pattern of changes was revealed in both groups, but TS-induced differences also became apparent: firstly, in TS-exposed animals from both experiments, a higher proportion of the labelled population was ciliated; secondly, a marginally larger labelled mucous-secreting cell population (transitional and mucous cells) was observed in the TS-exposed animals.

These data suggest pathways of differentiation within airway epithelium and illustrate responses to irritation by TS.

4.6. SUMMARY

Inhalation of TS by male SPF rats, studied in 2 sets of experiments, induced alterations in epithelial morphology, cell concentration and patterns of proliferation and differentiation.

Cell Numbers

1. The morphological changes comprised epithelial thickening and alterations to the granular content of secretory cells. In both

experiments, the proportion of serous cells decreased whereas that of mucous and transitional cells increased, with a shift from densely stained to weakly stained intracellular granules.

2. When compared to unexposed 14 day control animals TS was also found to induce changes in the non-secretory cell population: in both experiments total cell, ciliated and basal cell numbers increased whereas indeterminate cell numbers did not change in one set of experiments (2&4) and decreased in the other (1&3).

Proliferation

3. In Experiment 2, the size of the total proliferating cell population (ie. numbers of cells labelled with ^{3}H-T) decreased with time of TS exposure to significantly below the starting value at 14 days. In Experiment 1, the 3 day level of labelling, which was high compared the same time in Experiment 2, had decreased significantly by 14 days. A significant difference in levels of labelling between experiments was observed.

4. In Experiment 2, TS exposure produced a significant increase in labelled transitional cells (3 days) and then a decrease by 14 days in the concentration of labelled transitional, indeterminate and basal cells. In Experiment 1, only labelled transitional and indeterminate cells changed significantly: their numbers decreased from the high 3 day level, by 7 and 14 days respectively.

5. Consideration of changes in the relative contribution of each

labelled cell type to the proliferating population in each experiment suggests that in Experiment 1, TS exposure induces a proliferative response in the indeterminate, transitional and mucous cell compartments, whereas in Experiment 2, a much smaller proliferative response occurs, in the indeterminate and transitional cell compartments.

Differentiation

6. In both experiments, changes in a group of ^{3}H-T labelled cells were followed quantitatively during 14 days of TS exposure: the total number of labelled cells decreased substantially in Experiment 4 and remained constant in Experiment 3.

7. There were significant changes in labelled cell numbers in both experiments: labelled ciliated and transitional cells increased and serous and basal cells decreased.

8. Changes in the proportional contribution of each cell type to the total labelled cell population with time show that the proportion of basal cells decreased throughout concurrent with an increase in the proportional contribution of labelled ciliated, transitional and mucous cells. The contribution of labelled serous cells remained steady in Experiment 4 but decreased to zero in Experiment 3. Comparison with control values shows that 14 days of TS exposure produces a greater contribution from ciliated and secretory cells to the final total labelled population.

CHAPTER 5 - THE EFFECTS OF ORAL N-ACETYL CYSTEINE ON THE RESPONSE OF AIRWAY EPITHELIUM TO TOBACCO SMOKE

5.1. INTRODUCTION

Chapter 5 describes the results of experiments designed to investigate the effect of the mucolytic drug N-acetylcysteine (Nac) on TS-induced changes to cell proliferation and differentiation in airway epithelium.

The 68 experimental animals were divided into 2 major groups: 55 were used in proliferation experiments and 13 in differentiation experiments. Both groups comprised 4 sub-divisions according to treatment: control, Nac-only, TS-only and Nac+TS (see Table 2.3). Details of the dose and administration of Nac are described in Chapter 2, Section 2.2.4. The method of TS exposure was almost identical to that used in the proliferation and differentiation studies and is described in Chapter 2, Section 2.2.1. The one difference lay in the use of Capstan Full Strength cigarettes, as the tar and nicotine levels of the formerly used 'John Players Navy Cut Plain' had been decreased by the manufacturer. Animals from each group were killed on days 0, 1, 3, 7, 10 and 14 of the experiment and tissue samples from main bronchus and ileum were removed and processed (Chapter 2, Sections 2.2.3., 2.3. and 2.4). Epithelial cells were identified by morphological criteria (Chapter 3, Section 3.3) as **ciliated**, **serous**, **mucous**, **indeterminate** and **basal** cells. The **transitional** secretory cell type, containing both dense and lucent granules, was divided into two groups (**sero-mucous** and **muco-serous**) according to which type of granule was predominant. Numbers of all cells present were counted on days 0 and 14 only; numbers of labelled cells present were counted at each time point.

5.2. ANIMAL WEIGHTS

Fig 5.1 shows that animals in all groups gained weight during the experimental procedures and that the final mean weight of each treated group was significantly different from that of the control group.

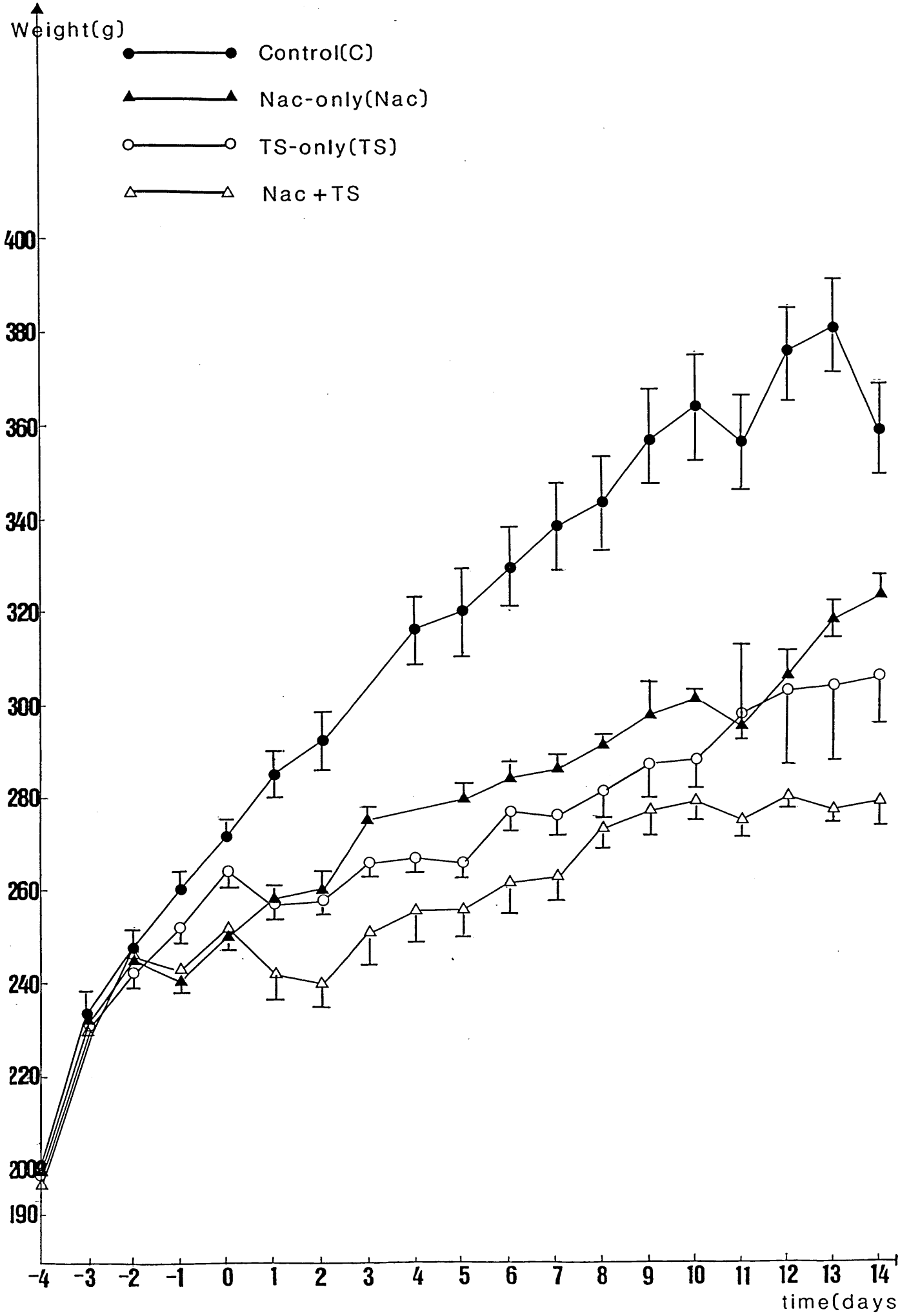
5.3. CELL TYPES: NUMBER AND PROPORTIONS

5.3.1. Total cell number

The mean value of the total cell number per 10 HPF in the proliferation experiments was 223.3 (± 3.6) in the control group of 5 animals at the beginning of the experiment (ie. day 0). After 14 days the values for control and treated groups were: **control**, 200.3 (± 2.4), **Nac-only**, 220.9 (± 3.5), **TS-only**, 238.5 (± 6.0), and **Nac+TS**, 246.9 (± 2.2).

Between days 0 and 14, a small (10 %) but significant reduction in the total number of cells per HPF occurred in the untreated control, but not the Nac-only group. As previously shown (Chapter 4, Section 4.3) TS induced a significant increase ($p<0.001$) in this parameter over the control day 14 value; an increase was also observed in the Nac+TS group.

FIGURE 5.1 ANIMAL WEIGHTS - EXPERIMENT 5



5.3.2. Number and proportions of each cell type

The proportions of each epithelial cell type in the control day 0 group were as follows: ciliated cells 49% (± 0.6%), serous cells 16% (± 0.7%), mucous cells 0.3% (± 0.08%), transitional cells 0.1% (± 0.05%), indeterminate cells 15% (± 0.5%), basal cells 20% (± 0.5%). The cell concentrations for each experimental group are displayed in Table 5.1. The statistical significance of the differences between proliferation treated and control groups at 14 days is shown in Table 5.2. The data indicates that the following changes occurred:

Ciliated cells: Although Nac caused no significant change in ciliated cell numbers, TS induced a significant increase ($p<0.001$) which was maintained and slightly but significantly enhanced when Nac was given at the same time as TS ($p<0.02$).

Serous cells in the control group decreased dramatically between 0 and 14 days and were present in only small numbers in all other groups. Comparisons at 14 days revealed no significant alterations due to Nac-only, TS-only, or to the two agents combined.

Mucous cells in the control group increased considerably between 0 and 14 days. Subsequent comparison of the latter value with those found in 14 day Nac-only, TS-only and Nac+TS animals showed a significant increase ($p<0.001$ for each group). The greatest was in the TS-only group, and the smallest in the group given Nac+TS: the difference between the latter groups was significant ($p<0.001$).

Transitional cells: Whilst Nac and TS caused no significant

TABLE 5.1

PROLIFERATION - Epithelial Cell Numbers/10 HPF (± SEM)

CELL TYPE	TREATMENT (days)				
	Control (0)	Control (14)	NAC-Only (14)	TS-Only (14)	NAC + TS (14)
CILIATED	110.1 (± 1.9)	105.4 (± 1.3)	107.9 (± 1.9)	133.8 (± 2.2)	140.9 (± 1.6)
SEROUS	36.7 (± 1.9)	0.1 (± 0.03)	5.0 (± 1.5)	2.4 (± 1.6)	1.1 (± 0.9)
MUCOUS	0.6 (± 0.2)	35.7 (± 1.7)	47.9 (± 3.6)	51.7 (± 2.3)	44.7 (± 1.3)
TRANSITIONAL sero-mucous	0.12 (± 0.06)	0.03 (± 0.02)	0.02 (± 0.02)	0.02 (± 0.02)	0.27 (± 0.1)
muco-serous	0.23 (± 0.12)	0.06 (± 0.03)	0.08 (± 0.05)	0.14 (± 0.11)	1.5 (± 0.3)
INDETERMINATE	32.0 (± 1.0)	19.6 (± 1.2)	18.6 (± 1.1)	4.0 (± 0.6)	5.3 (± 0.6)
BASAL	44.2 (± 1.4)	39.5 (± 0.9)	41.6 (± 0.9)	51.3 (± 1.1)	53.4 (± 1.1)

TABLE 5.2

PROLIFERATION - SIGNIFICANT CHANGES IN:

Epithelial Cell Numbers/10 HPF

14 days of Treatment compared with 14 day Controls

	TREATMENT		
CELL TYPE	NAC-Only (14 days)	TS-Only (14 days)	NAC + TS (14 days)
CILIATED	NS	↑ $p < 0.001$	↑ $p < 0.001$
SEROUS	NS	NS	NS
MUCOUS	↑ $p < 0.001$	↑ $p < 0.001$	↑ $p < 0.001$
TRANSITIONAL sero-mucous	NS	NS	↑ $p < 0.01$
muco-serous	NS	NS	↑ $p < 0.001$
INDETERMINATE	NS	↓ $p < 0.001$	↓ $p < 0.001$
BASAL	NS	↑ $p < 0.001$	↑ $p < 0.001$

NS = Not significant.

change in the number of transitional cells, together the agents produced similar and significant increases above the 14 day control values ($p<0.01$).

Indeterminate cells in the control group decreased significantly between 0 and 14 days. Comparison between 14 day control and treated groups showed that Nac did not induce further significant change but TS, alone and in combination with Nac, caused similar significant decreases ($p<0.001$), ie. Nac did not influence the TS-induced effect.

Basal cells: There was no significant change in the number of basal cells following Nac, but both TS and Nac+TS produced significant and similar increases above the 14 day control values ($p<0.001$).

5.4. PROLIFERATION

5.4.1. Total Number of Labelled Cells

In the control group there was no significant difference in the total number of labelled cells between days 0 and 14. Therefore the values were combined and used as the control day 0 point. In each treatment group, the number of labelled epithelial cells per 10 HPF was counted and plotted against time of TS exposure (Fig. 5.2. a&b). Fig. 5.2a shows the proliferative response in animals exposed to 14 days of TS-only, compared with that in animals given Nac+TS for the same time.

FIGURE 5.2 PROLIFERATION:
TOTAL LABELLED EPITHELIAL CELLS/10 HPF (± SEM) VS TIME

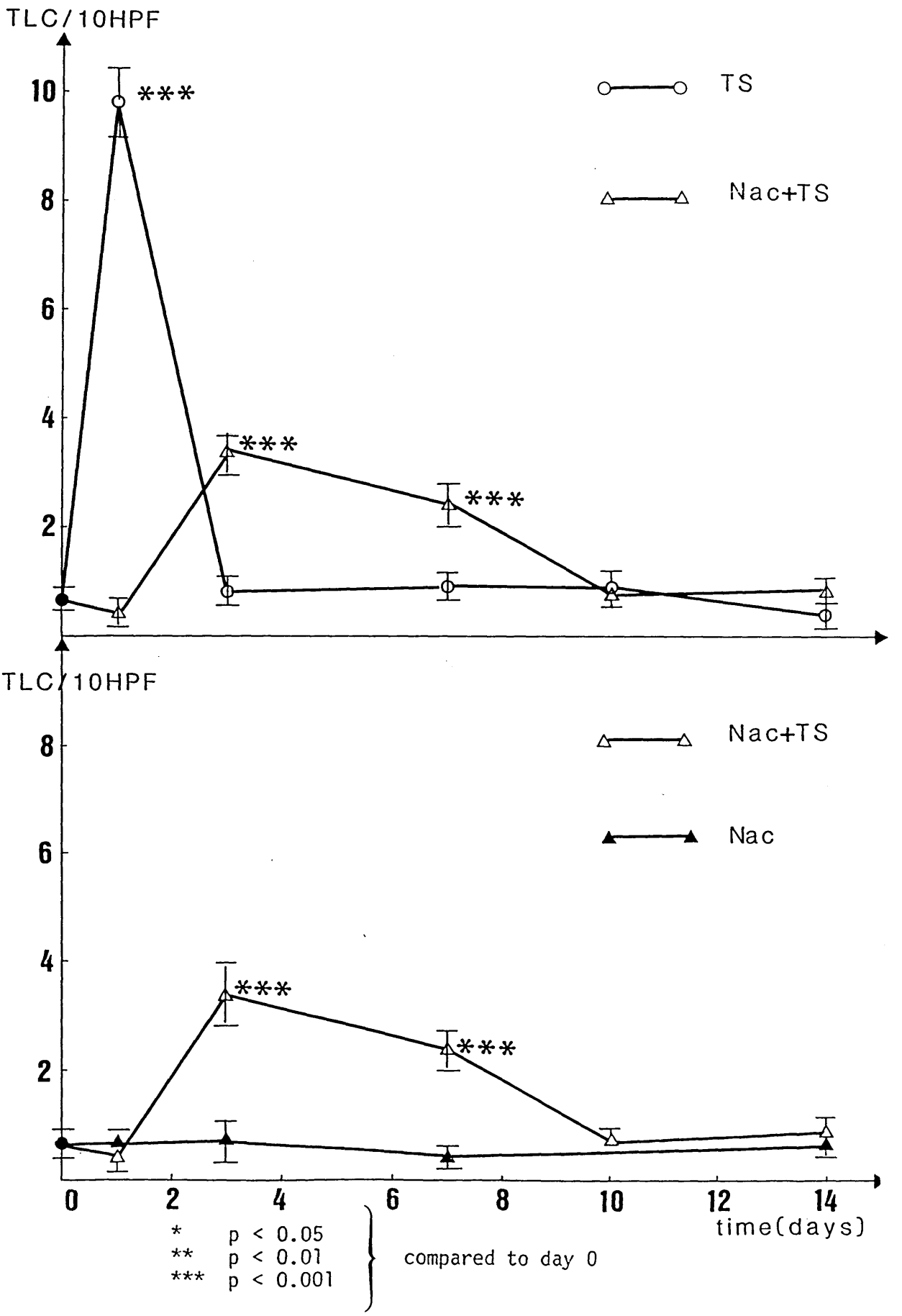


Fig. 5.2b compares the proliferative response in the group given Nac+TS with that receiving Nac-only. The results show that:

1. Nac caused no significant change in the number of labelled cells.

2. TS induced a large and highly significant increase ($p<0.001$) in the number of labelled cells, which peaked at 24 hours, returned to the control value by 3 days and remained at or near the control value for the duration of the experiment.

3. The combination of Nac and TS had 3 effects on the peak of labelled (ie. proliferating) cells: it was (i) delayed until 3 days, (ii) greatly reduced in size, and (iii) prolonged at a lower level, until its return to the control value at 10 days of TS exposure (Fig. 5.2b).

4. By measurement of the area under the curve 'Total Labelled Cells vs. Time' (Fig 5.2a&b), an estimate of the aggregate total number of cells per 10 HPF which became labelled during the 14 days can be obtained. The area under the TS curve was slightly but significantly larger than that of Nac+TS ($p<0.05$), suggesting that more cells proliferated in the TS-only group than in the group given Nac+TS.

5.4.2. Number of Each Cell Type Labelled.

The concentrations and proportions of labelled cells in the control group are shown in Table 5.3 (a&b). Comparison between the number of each labelled cell type on days 0 and 14 (Table 5.3) showed

TABLE 5.3 a)

CONTROL PROLIFERATION - Labelled Epithelial Cells/10 HPF (± SEM)

CELL TYPE	TIME (days) 0	14	
CILIATED	0.0	0.01 (± 0.01)	NS
SEROUS	0.17 (± 0.06)	0.0 *	
MUCOUS	0.0	0.06 (± 0.03)	NS
TRANSITIONAL	0.0	0.0	
INDETERMINATE	0.13 (± 0.08)	0.03 (± 0.02)	NS
BASAL	0.37 (± 0.08)	0.56 (± 0.09)	NS
TOTAL	0.67 (± 0.1)	0.66 (± 0.1)	NS

NS = Not Significant.
* $p < 0.05$.

TABLE 5.3 b)

CONTROL PROLIFERATION - % Labelled Epithelial Cells + SEM.

CELL TYPE	TIME (days) 0	14	
CILIATED	0	0.01 (± 0.01)	NS
SEROUS	0.4 (± 0.1)	0	**
MUCOUS	0	0.16 (± 0.08)	NS
TRANSITIONAL	0	0	
INDETERMINATE	0.35 (± 0.2)	0.20 (± 0.1)	NS
BASAL	0.83 (± 0.2)	1.5 (± 0.2)	*
TOTAL	0.30 (± 0.07)	0.33 (± 0.06)	NS

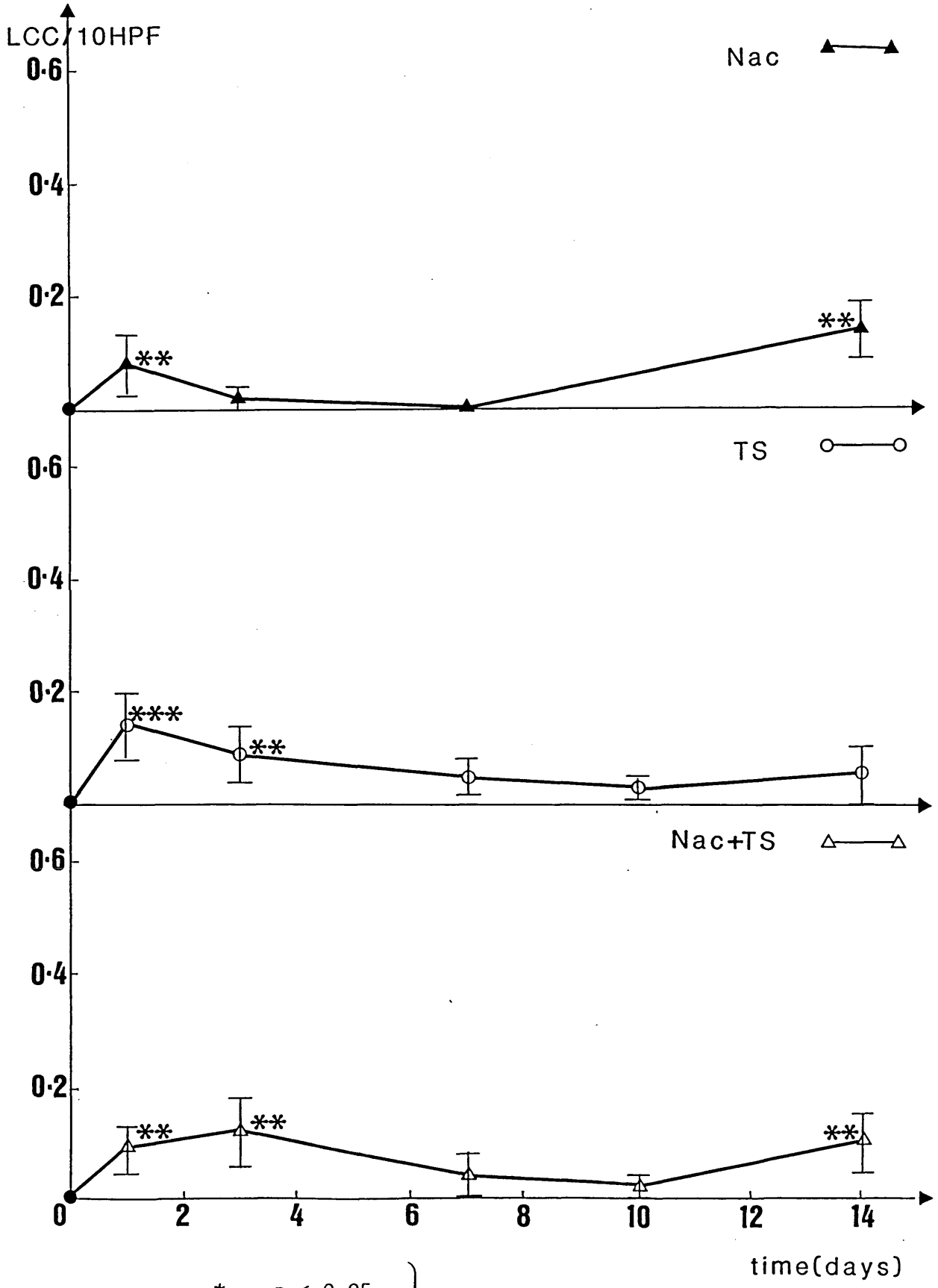
that only serous cells were significantly different ($p<0.002$). Thus for all except serous cells, the values were combined and plotted as the day 0 value.

The number of each labelled cell type per 10 HPF for each treatment group was plotted against time of TS exposure (Figs 5.3-5.8); these data are shown in Appendix 1, Tables A.1.1-A.1.5. The significant changes occurring with time for each cell type in each group are shown in Tables 5.4-5.9.

Ciliated cells: The number of labelled ciliated cells observed during the experiment was always very small. Whilst there were no significant differences between groups, at any time point, within each treatment group significant changes did occur with time (Fig. 5.3). In TS-only animals, labelled ciliated cells appeared at 24 hours ($p<0.02$), but their numbers decreased to control values, (ie. not significantly different from zero) by 14 days of TS exposure. In both Nac-only and Nac+TS groups, significant numbers of labelled ciliated cells were present at 24 hours ($p<0.02$); they decreased to control values as TS exposure progressed, then increased significantly by 14 days ($p<0.02$) (Table 5.4).

Serous cells: There were significant changes in the concentration of labelled serous cells in the treated animals (Fig. 5.4). In the Nac-only group there was no change until a decrease occurred at 14 days ($p<0.01$). The concentration of labelled serous cells increased in the TS-only group at 3 days ($p<0.01$) and the Nac+TS group ($p<0.001$) at 7 days; there was no significant difference between the 7 day labelling levels in the 2 groups. By 14 days the number of

FIGURE 5.3 PROLIFERATION:
LABELLED CILIATED CELLS/10 HPF (± SEM) VS TIME



* p < 0.05
** p < 0.01
*** p < 0.001

compared to day 0

TABLE 5.4

PROLIFERATION - SIGNIFICANT CHANGES IN:

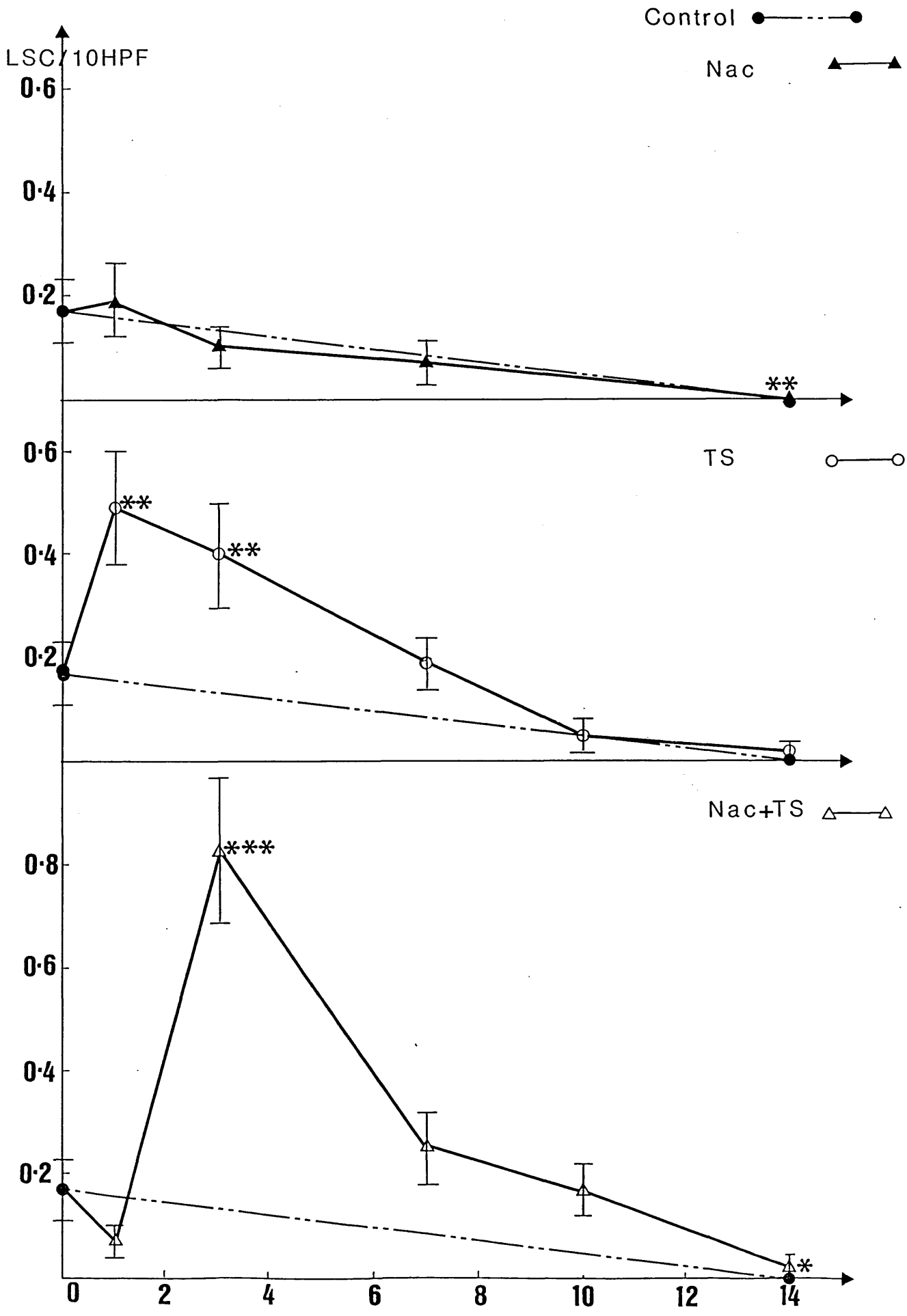
Labelled Ciliated Cells/10 HPF

Each time point compared with day 0 Controls

	TIME (days)				
TREATMENT	1	3	7	10	14
NAC-Only	↑$p < 0.02$	NS	NS	--	↑$p < 0.01$
TS-Only	↑$p < 0.002$	↑$p < 0.02$	NS	NS	NS
NAC + TS	↑$p < 0.02$	↑$p < 0.02$	NS	NS	↑$p < 0.02$

NS = Not Significant.
-- = No samples taken.

FIGURE 5.4 PROLIFERATION:
LABELLED SEROUS CELLS/10 HPF (± SEM) VS TIME



* p < 0.05
** p < 0.01
*** p < 0.001
} compared to day 0

TABLE 5.5

PROLIFERATION - SIGNIFICANT CHANGES IN:

Labelled Serous Cells/10 HPF

Each time point compared with day 0 Controls

	TIME (days)				
TREATMENT	1	3	7	10	14
NAC-Only	NS	NS	NS	--	↓p < 0.01
TS-Only	↑p <0.01	↑p < 0.05	NS	NS	NS
NAC + TS	NS	↑p < 0.001	NS	NS	↓p < 0.05

NS = Not Significant.
-- = No samples taken.

labelled serous cells in the TS-only and Nac+TS groups fell to below control levels (Table 5.5).

Mucous Cells: (Fig. 5.5) There were no labelled mucous cells observed in animals given Nac until 14 days. TS-exposed animals showed a significant increase in mucous cell labelling by 10 days ($p<0.001$) which was maintained at 14 days. Labelled mucous cells in the group given Nac and TS showed a significant increase at 3 days ($p<0.001$) which was maintained until 10 days, and increased further by 14 days ($p<0.02$) (Table 5.6). Comparisons between all groups at 14 days showed that the Nac+TS animals had significantly more labelled mucous cells by 14 days than any other group.

Transitional Cells: Sero-mucous cells showed no significant changes in labelling within or between any experimental group. Muco-serous cells showed significant changes only in the group exposed to both Nac and TS: a small significant increase ($p<0.02$) in labelling occurred at 3 days which was maintained until 7 days and then fell to the control level by 10 days (Fig. 5.6) (Table 5.7).

Indeterminate cells: (Fig 5.7) Neither Nac-only, TS-only nor Nac+TS groups showed any significant changes in the concentration of labelled indeterminate cells with time, but there was a significant difference between Nac+TS and the other treated groups at 24 h.

Basal Cells: (Fig 5.8) Nac had no effect on the concentration of labelled basal cells; TS induced a 20 fold increase in labelling ($p<0.001$) at 24 hours which had returned to control values by 3 days. Animals given Nac+TS showed a significantly smaller increase which

FIGURE 5.5 PROLIFERAION:
LABELLED MUCOUS CELLS/10 HPF (± SEM) VS TIME

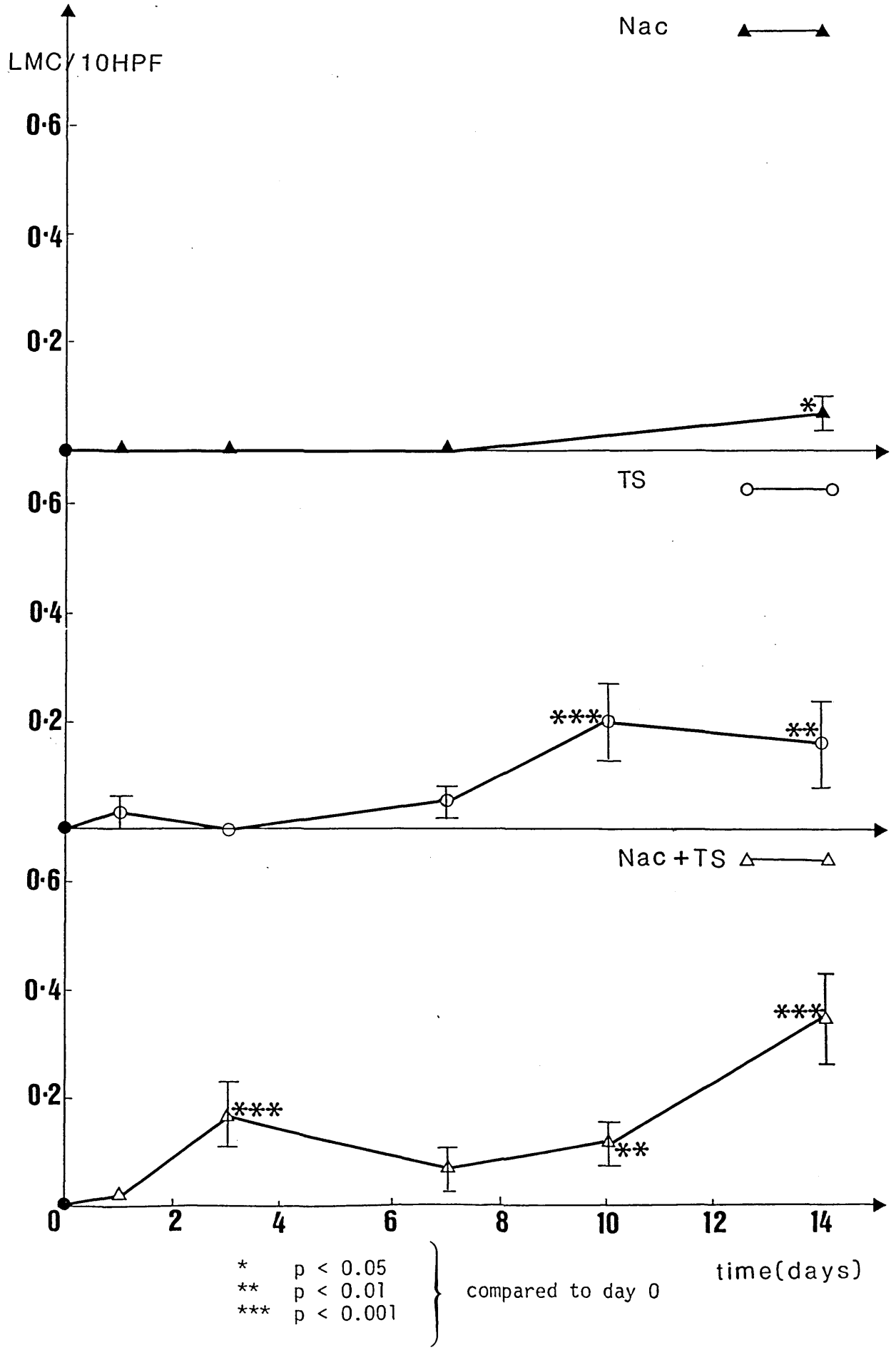


TABLE 5.6

PROLIFERATION - SIGNIFICANT CHANGES IN:

Labelled Mucous Cells/10 HPF

Each time point compared with day 0 Controls

	TIME (days)				
TREATMENT	1	3	7	10	14
NAC-Only	NS	NS	NS	--	↑ $p < 0.05$
TS-Only	NS	NS	NS	↑ $p < 0.001$	↑ $p < 0.01$
NAC + TS	NS	↑ $p < 0.001$	NS	↑ $p < 0.01$	↑ $p < 0.001$

NS = Not Significant.
-- = No samples taken.

FIGURE 5.6 PROLIFERATION:
Nac + TS: LABELLED TRANSITIONAL CELLS/10 HPF (± SEM) VS TIME

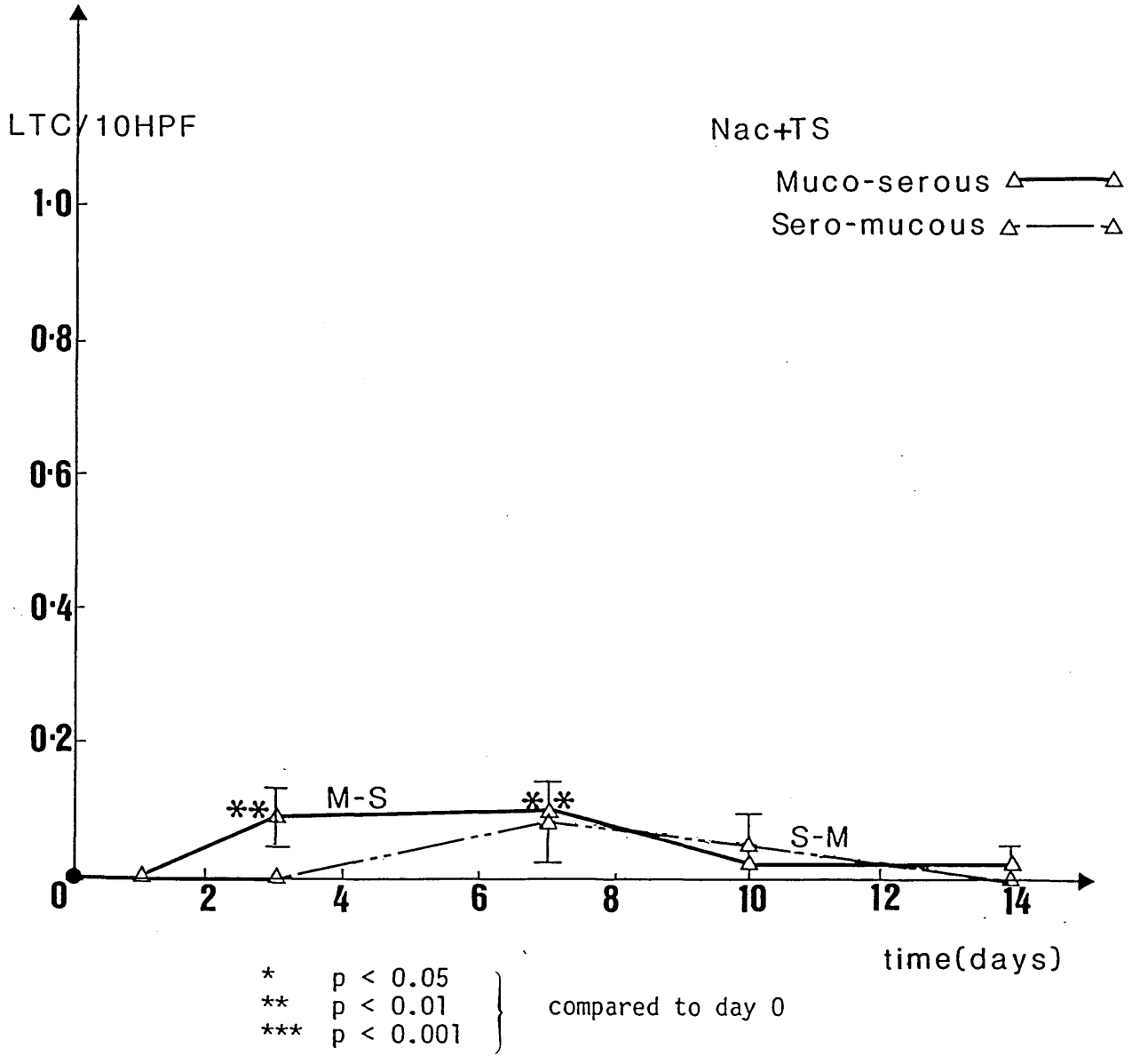


TABLE 5.7

PROLIFERATION - SIGNIFICANT CHANGES IN:

Labelled Transitional Cells/10 HPF

Each time point compared with day 0 Controls

	TIME (days)				
TREATMENT	1	3	7	10	14
Sero-Mucous Cells:					
NAC-Only	0	0	0	--	0
TS-Only	NS	NS	NS	NS	NS
NAC + TS	NS	NS	NS	NS	NS
Muco-Serous Cells:					
NAC-Only	0	0	0		0
TS-Only	NS	NS	NS	NS	NS
NAC + TS	NS	↑$p < 0.02$	↑$p < 0.02$	NS	NS

NS = Not Significant.
0 = No labelled cells observed.
-- = No samples taken.

FIGURE 5.7 PROLIFERATION:
LABELLED INDETERMINATE CELLS/10 HPF (± SEM) VS TIME

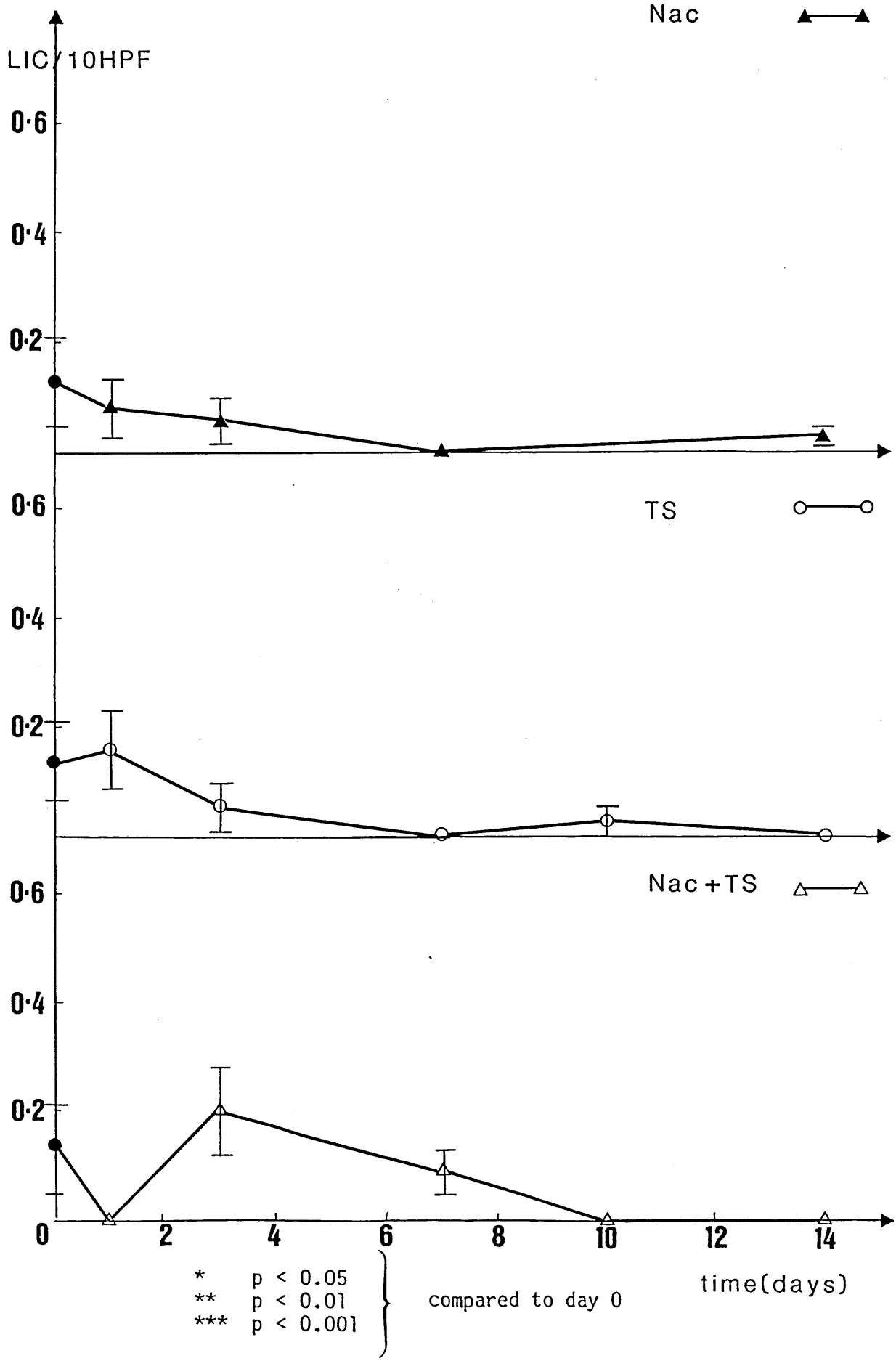


FIGURE 5.8 PROLIFERATION:
LABELLED BASAL CELLS/10 HPF (± SEM) VS TIME

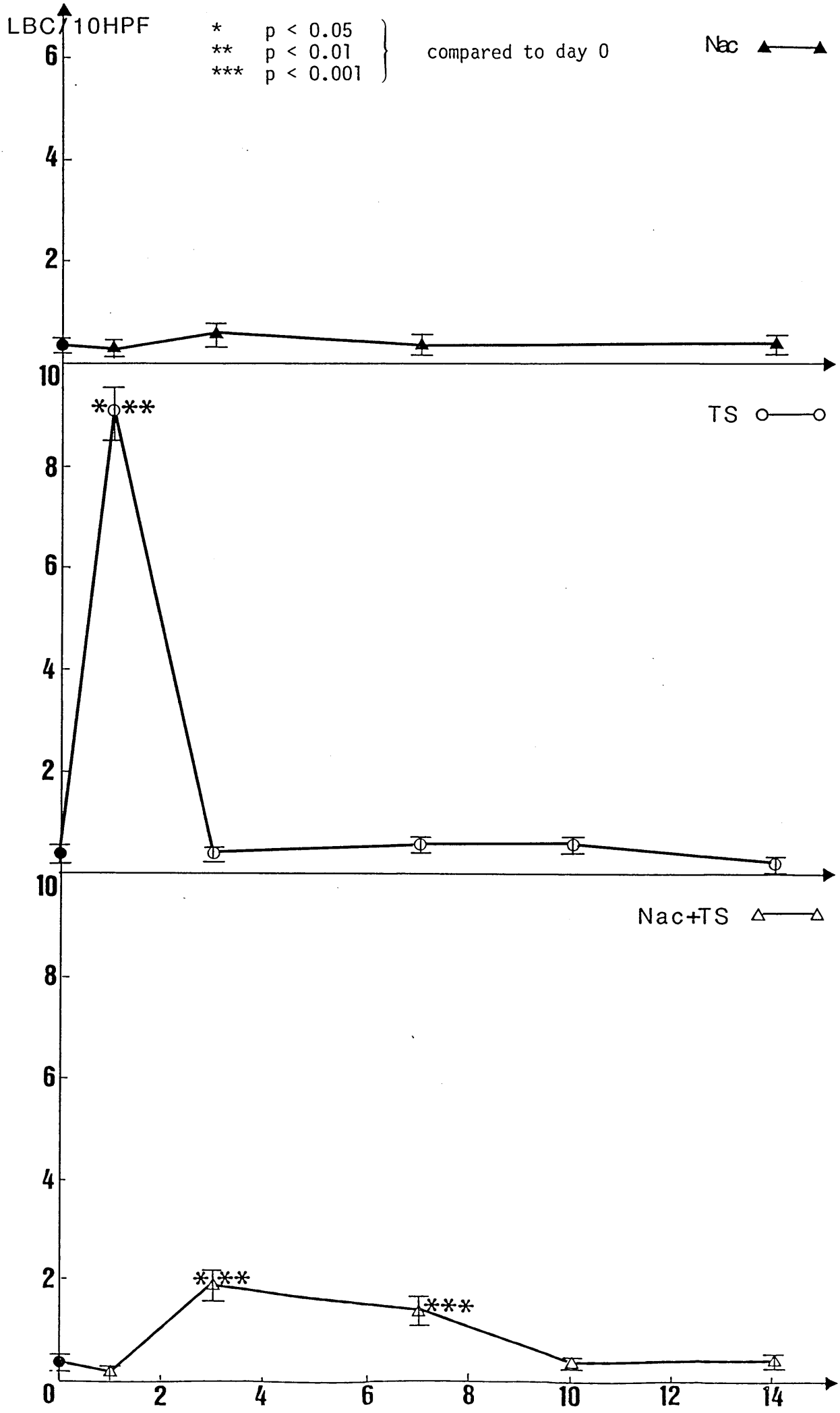


TABLE 5.8

PROLIFERATION - SIGNIFICANT CHANGES IN:

Labelled Basal Cells/10 HPF

Each time point compared with day 0 Controls

	TIME (days)				
TREATMENT	1	3	7	10	14
NAC-Only	NS	NS	NS	--	NS
TS-Only	↑ $p < 0.001$	NS	↑ $p < 0.05$	NS	NS
NAC + TS	NS	↑ $p < 0.001$	↑ $p < 0.001$	NS	NS

NS = Not Significant.
-- = No samples taken.

occurred later that of the TS-only group (p 0.001), was maintained until 7 days, then returned to the control value by 10 days (Table 5.8)

5.4.3. Percentage Label Distribution

To assess the contribution of each cell type to the proliferative response, the number of each labelled cell type was expressed as a percentage of the total number of labelled cells present (Tables 5.9-5.12). This data shows that:

1. In control animals, basal, serous, and indeterminate cells were labelled (ie. proliferating) and therefore part of the growth fraction (Table 5.9).

2. During TS exposure, basal cells played a major role in the large, early proliferative response: on day 1 of exposure, 92% of all labelled cells were basal (Table 5.11).

3. With time of TS exposure, mucous cells proliferated and became a relatively large component of the response: by day 14, 40% of the labelled cells in both TS and Nac+TS groups were mucous (Tables 5.11 & 5.12).

4. Addition of Nac during exposure to TS appeared to change the basal cell response: in the Nac+TS group 50% of the labelled cells were basal on day 1. However the response of mucous cells did not change: by day 14, mucous cells were still 42% of the labelled cell population in the Nac+TS group (Table 5.12).

TABLE 5.9

CONTROL PROLIFERATION - % Label Distribution

CELL TYPE	TIME (days) 0	14
CILIATED	0	3
SEROUS	31	0
MUCOUS	0	8
TRANSITIONAL	0	0
INDETERMINATE	11	4
BASAL	58	85

TABLE 5.10

NAC-ONLY - PROLIFERATION

% Label Distribution

	TIME (days)				
CELL TYPE	1	3	7	10	14
CILIATED	13	5	0	-	21
SEROUS	32	21	21	-	0
MUCOUS	0	0	0	-	10
TRANSITIONAL	0	0	0	-	0
INDETERMINATE	13	10	0	-	5
BASAL	42	64	79	-	66

TABLE 5.11

TOBACCO SMOKE-ONLY - PROLIFERATION

% Label Distribution

	TIME (days)				
CELL TYPE	1	3	7	10	14
CILIATED	2	9	4	4	12
SEROUS	5	40	18	10	3
MUCOUS	0	0	8	19	40
TRANSITIONAL	0	1	7	8	1
INDETERMINATE	1	9	0	3	0
BASAL	92	41	68	59	50

TABLE 5.12

NAC + TOBACCO SMOKE - PROLIFERATION

% Label Distribution

	TIME (days)				
CELL TYPE	1	3	7	10	14
CILIATED	25	3	1	3	12
SEROUS	18	21	14	21	1
MUCOUS	7	12	4	17	42
TRANSITIONAL	0	3	10	5	1
INDETERMINATE	0	4	3	0	0
BASAL	50	57	68	54	42

5. In both control, Nac-only and Nac+TS groups the percentage of labelled cells which were serous decreased with time of TS exposure, whereas fluctuation was observed in the TS-only group.

6. Ciliated cells became a substantial part of the labelled cell population in the Nac-only, TS-only and Nac+TS groups (Tables 5.10 - 5.12).

5.5. DIFFERENTIATION

To observe possible pathways of differentiation in each of the treatment groups, ^{3}H-T was administered as a single pulse on day 0 in order to label a group of cells such that the label was distributed between them in proportion to the number of each cell type synthesizing DNA. No further ^{3}H-T was added during the experiment. Changes in the pattern of labelling between days 0 and 14 days in each experimental group suggest pathways of differentiation, changes induced by TS and modification of these changes, if any, by Nac.

5.5.1. Total Number of Labelled Cells

The mean values for the total number of labelled cells per 10 HPF for the control group (day 0) and for each group at 14 days of TS exposure are shown in Table 5.13. The only significant difference is between the 14 day control group value and all other groups ($p < 0.001$).

5.5.2. The Number of Labelled Cell Types and Percentage Label Distribution

The number of each cell type labelled was quantified on days 0 and 14 in the control group and on day 14 only for treated groups (Table 5.13); the data was then expressed as a percentage of the total number of labelled cells (Table 5.14). Changes in this distribution suggest normal pathways of differentiation and alterations due to TS exposure and drug treatment. The data demonstrates that in:

Control animals: The contribution of both ciliated and mucous cells to the labelled cell population increased with time and that of labelled basal cells decreased, while the proportions of the labelled population which were serous and indeterminate were the same on days 0 and 14.

Nac-Only: As in the control group, the proportion of labelled cells which were ciliated and mucous increased with time. In contrast to the control group however, there was no reduction in the proportion of labelled basal cells and that of labelled serous cells decreased.

TS-Only: At 14 days the group exposed to TS showed a rise in the percentage of labelled ciliated cells, four times greater than that seen in the control and Nac-only groups. When compared with control values, the changes in the proportions of labelled serous, transitional and indeterminate cell types were small. No labelled cells were mucous at this time. The percentage of labelled cells which was basal showed a decrease similar to that seen in control animals.

TABLE 5.13

DIFFERENTIATION - Labelled Epithelial Cell Numbers/10 HPF (± SEM)

CELL TYPE	TREATMENT (days) Control (0)	Control (14)	NAC-Only (14)	TS-Only (14)	NAC + TS (14)
CILIATED	0	0.12** (± 0.04)	0.04 (± 0.03)	0.25 (± 0.08)	0.13 (± 0.07)
SEROUS	0.17 (± 0.06)	0.4** (± 0.09)	0.06*** (± 0.04)	0.2* (± 0.07)	0.17* (± 0.07)
MUCOUS	0	0.17** (± 0.06)	0.12 (± 0.05)	0**	0.06 (± 0.03)
TRANSITIONAL sero-mucous	0	0	0	0.02 (± 0.02)	0.02 (± 0.02)
muco-serous	0	0	0	0	0.02 (± 0.02)
INDETERMINATE	0.13 (± 0.08)	0.09 (± 0.04)	0.04 (± 0.03)	0.04 (± 0.03)	0.08 (± 0.04)
BASAL	0.37 (± 0.09)	0.29 (± 0.06)	0.28 (± 0.09)	0.13* (± 0.05)	0.20 (± 0.08)
TOTAL	0.67 (± 0.15)	1.07** (± 0.14)	0.52** (± 0.1)	0.56** (± 0.13)	0.69** (± 0.15)

** $p < 0.01$ compared to Control day 0

* $p < 0.05$ compared to
** $p < 0.01$ Control
*** $p < 0.001$ day 14

TABLE 5.14

DIFFERENTIATION:

% Label Distribution

GROUP	CELL TYPE					
	Ciliated	Serous	Mucous	Transitional	Indeterminate	Basal
CONTROL - day 0	0	31	0	0	11	58
CONTROL - day 14	11	35	15	0	10	27
NAC-Only	9	9	21	0	9	52
TS-Only	41	31	0	3	6	22
NAC + TS	20	21	12	3	13	31

Nac + TS: In this group the increase in the percentage of labelled cells which were ciliated was half that observed in the TS-only group and closer to that of the 14 day controls. The increase in the percentage of labelled mucous cells was also equivalent to that of controls.

5.6. SUMMARY

Cell Numbers

1. The TS-induced changes to the numbers of each cell type per unit length epithelium and the modifying influence of Nac are as follows:

CELL TYPE	TS-INDUCED EFFECT	MODIFICATION OF TS EFFECT BY Nac
ciliated	↑($p < 0.001$)	potentiates (68%)
mucous	↑($p < 0.001$)	inhibits (20%)
transitional	no effect	↑($p < 0.01$)
indeterminate	↓($p < 0.001$)	no effect
basal	↑($p < 0.001$)	no effect
total	↑($p < 0.001$)	no effect

2. There was no significant difference, for serous cells, between the control 14 day value and any treatment group.

Proliferation

3. Nac had 5 effects on the TS-induced increase in the total numbers of labelled cells: (i) the proliferative peak was delayed from day 1 to

day 3 of TS exposure; (ii) the amplitude of the increase was reduced; (iii) the attenuated increase was of a longer duration; (iv) the aggregate proliferating population was decreased and (v) Nac modified the basal cell response but did not alter that of the mucous cell.

4. The table below summarizes the TS-induced proliferative changes for each cell type and the modification due to added Nac:

LABELLED CELL TYPE	TS-INDUCED EFFECT	MODIFICATION BY NAC
ciliated	NS	NS
serous	↑ $p<0.01$	delayed increase
mucous	↑ $p<0.001$	earlier ↑ + extra ↑ @ 14 days
transitional	NS	no significant effect
indeterminate	NS	no significant effect
basal	↑ $p<0.001$	delayed & prolonged
total	↑ $p<0.001$	delays & prolonged

NS = no significant change

5. The major effect of Nac was exerted on proliferating basal cells

which mirrored that observed in the total proliferating population.

6. When the number of each cell type labelled was expressed as a percentage of the total numbers of labelled cells, basal cells were shown to be the major component of the large early response to TS. Subsequently, mucous cells became a significant proportion of the proliferating population. Nac modified the basal cell response but did not alter that of the mucous cell.

Differentiation

7. Following a single pulse of ^{3}H-T in control animals both ciliated and mucous cells became labelled with time at the expense of the basal cell population which was initially labelled.

8. With Nac-only the pattern of increased labelling was, for ciliated and mucous cells, similar to that of untreated controls, but at the expense of serous rather than basal cell labelling.

9. In the TS-only group a progressive increase in labelling of ciliated cells to a level at 14 days four-fold greater than that of the controls at the same time, was observed. This increase was accompanied by a decrease in the labelling in basal cells.

10. When Nac was given to animals with TS, the increase in the percentage of label in ciliated cells was halved compared to that resulting from TS-only and the pattern of labelling (ie. differentiation) returned towards that of the controls.

CHAPTER 6 - RESULTS: SYNTHESIS AND COMMENT

Each of the three experimental sets (Proliferation experiments 1, 2 and 5; Differentiation experiments 3, 4,and 5) shows a response to TS exposure composed of 1) metaplasia, from serous to mucous cells via the formation of transitional cells; 2) proliferation, of indeterminate, transitional and mucous cells in Experiment 1 and mainly basal cells in Experiment 5; and 3) changes in the pathways of differentiation such that basal and serous cells differentiated more rapidly and more ciliated and transitional cells were produced, than in control animals. The features of each set of experimental results which lead to the above conclusions will be now be briefly examined. Particularly important for the interpretation, are the percentages of each cell compartment labelled, which enable the proliferative activity of individual cell groups to be monitored.

PROLIFERATION

Experiment 1

1. 14 days of TS exposure increased the proportion of epithelial mucous cells to 14% of the population. There were no control day 0 values in this experiment, thus the appropiate values from Experiments 2 and 5, 0.3% in both experiments, were used for comparison (see **Table 3.2** and **Chapter 5.3.2.**).

2. Compared to the control day 0 values from Experiments 2 and 5, the TS-exposed animals had a higher level of labelling from days 3-14.

3. In particular, at 3 days: 4.0% of indeterminate cells, 9.8% of transitional cells, 2.0% of mucous cells } **Table 4.4b)**

   were labelled compared to the control day 0 values in Experiment 2 (0.6%, 0.1%, and 0.0% - **Table 3.3**) and Experiment 5 (0.4%, 0.0%, and 0.0% - **Table 5.3b**).

4. By 7 days of TS exposure, the labelling pattern was:

   1% of indeterminate cells
   0.0% of transitional cells } **Table 4.4b)**
   3% of mucous cells

   at which time mucous cells formed 40% of the labelled cell population.

5. By 14 days the labelled percentage of the mucous cell compartment (0.3%), formed 21% of the total labelled population and was higher than the 14 day control value from Experiment 2 (0%) but not significantly greater than that from Experiment 5 (0.2%); there were no transitional or indeterminate labelled cells. It was notable that the percentage of labelled basal cells changed little throughout the experimental period. **(Tables 3.3, 4.4b., 5.3b)**

6. Comparison of the numbers of transitional cells in this experiment, with control values for Experiment 2 shows that a small consistant increase occurred during the experiment concurrent with large decreases and increases in serous and mucous cells respectively. Thus it is possible that this compartment served as a 'through' compartment for serous cells undergoing metaplasia to mucous cells. **(Tables 3.2 and 4.2)**

Comment: In this experiment, mucous cell numbers increased substantially as a proportion of the epithelial population. TS exposure induced a wave of proliferation, which was very active by 3 days in the indeterminate and transitional compartments continuing at 7 days in the mucous cell compartment, where, at 14 days, labelled cells were still present. Basal cells did not participate in the response. It is probable that metaplasia of serous cells to mucous cells via transitional cells contributed to the increase in the mucous cell proportion.

Experiment 2

1. By contrast in this experiment, a very small increase (0.3-1.2%) in the proportion of mucous cells occurred; transitional cells also increased slightly as a proportion of the epithelial population **(Table 4.2)**

2. There was no significant difference between control and TS-exposed total labelled cell populations (**Fig 4.7**).

3. The transitional compartment was the only one in which a proliferative response to TS occurred: at 3 days 0.8% of these cells were labelled (significantly different from the control value of 0.1%), forming 12% of the labelled population, compared with 2% in controls (**Tables 3.3, 4.4b, 4.6**).

4. Thereafter the labelled percentage of indeterminate and basal as well as transitional compartments decreased, as did their relative contributions to the labelled cell population (**Table 4.6**).

Comment: When compared to control animals, this experiment showed a very small response to TS exposure in terms of both increase in mucous cell number and cell proliferation; it is likely that the increase in mucous cell number that did occur arose from serous cell metaplasia. These findings suggest that in this experiment the TS concentration to which the animals were exposed was low compared to that of Experiment 1 (see Chapter 7.4.1).

Experiment 5

1. Increases in mucous cell number occurred in both control and TS-exposed groups in the following order (**Table 5.1**):

TS-only < Nac+TS < Nac-only < Control-14days < Control-day0

2. The control values for labelled cell concentrations correspond to those of Experiment 2 (**Tables 4.4a and 5.3a**).

3. There was a major proliferative response to TS in the basal cell compartment, which peaked at 24h becoming 92% of the total labelled population (compared to 58% in control animals) and returned to control levels by 3 days. Mucous cells also proliferated (becoming 40% of the total labelled population by 14 days, compared to 8% in

the control group at 14 days), but later and at a much lower level than basal cells. Other compartments involved in the proliferative response were: serous, ciliated and transitional (**Figs 5.3 – 5.8**).

The effect of Nac on the TS-induced epithelial response is discussed later in Chapter 7.3.

Comment: The response to TS in this experiment was clearly very different to that in Experiments 1 or 2. The proliferative response was substantially greater and this time the major contributor was the basal cell compartment. However the mucous cell did participate and as in Experiment 1, became 40% of the total labelled cell population by 14 days. The difference in response between basal and mucous cells is interesting and may be indicative of a moderated epithelial reaction which is proportional to severity of the injury. The variation in the response between experiments suggests that different exposure conditions obtained in each case (see Chapter 7.4).

Differentiation

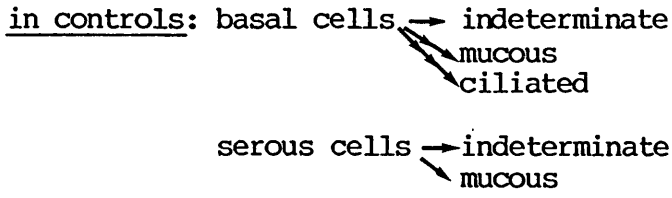
In the context of these experiments, differentiation is defined as the process of change from a stem cell, to a functional cell which can still proliferate, to a mature functional cell which has lost its ability to proliferate (ie. is terminally differentiated); the latter step may or may not involve division; metaplasia is change from one functional cell type to another functional cell type. For the purposes of comparison and discussion, maturation is defined here as change from a cell at an early stage of function to one at a fully functional stage which has terminally differentiated.

The processes of both differentiation and metaplasia were monitored by assessing changes in the proportions of each cell compartment that was labelled, with time. Decreases or increases in these proportions are spoken of as compartments 'losing' or 'gaining' label, processes which result from both differentiation and metaplasia. The relative contribution of these two processes to change in a particular compartment cannot be reliably distinguished in the data discussed herein.

Experiment 3

1. There was no significant difference in total number of labelled cells between the beginning and end of the experiment (**Fig. 4.12b**)

2. Comparison of data from **Tables 3.8 (control)** and **4.9 (TS-exposed)** shows that basal and serous cell compartments lost label and ciliated and secretory (transitional and mucous) compartments gained label in both groups; the indeterminate compartment gained label in the controls and gained then lost it in TS-exposed animals. These changes suggest that:



in TS-exposed epithelium:

a) the ciliated proportion of the total labelled population at 14 days was larger than in the controls;

b) the basal proportion of the labelled population became smaller earlier, suggesting that label moved out of this compartment more rapidly;

c) the size of the secretory proportion (serous, transitional and mucous cells) of the labelled cell population changed from the control value at 3 days (20%) to the 14 day values of both control

and TS-exposed groups, which are equivilent (33% and 31% respectively); the distributions within these proportions are different -

control (day 3): serous 20%, transitional 0%, mucous 0%
control (14 days): serous 13%, transitional 0%, mucous 18%
TS-exposed (14 days): serous 0%, transitional 26%, mucous 7%

Comment: these differences suggest that - in control animals, basal cells can give rise to indeterminate and ciliated cells (possibly via indeterminate cells) and may also contribute to the secretory cell population, by differentiation; TS accelerates the differentiation process, resulting in increased ciliated cell production; although there is an increase in the proportion of the total labelled cell population which is secretory between days 0 and 14, the similarity of the control and TS-exposed secretory proportions at this time suggests that TS exposure itself did not increase mucous cell numbers via differentiation pathways. However there are more labelled transitional cells, possibly due to metaplasia from serous cells.

Experiment 4

1. In this experiment the total number of labelled epithelial cells decreased considerably between days 0 and 14 in both control and TS-exposed groups. However the label distribution between cell compartments within the decreasing total was assessed and control and TS-exposed groups compared.

2. TS exposure induced the following differences in the differentiation pathways:

   a) loss of label from the basal cell compartment (differentiation) occurred earlier but at the end of the experiment the total loss of label was virtually the same in control and TS groups;

   b) by 14 days there was more label in the ciliated compartment: it formed 30% of the total labelled population, compared with 16% in controls, suggesting that more ciliated cells were produced, a finding common to the three differentiation experiments;

c) in the control day 0 group the secretory proportion of the total labelled cell population was 19%; by 14 days the total secretory compartment in the TS group held 42% of the epithelial label, compared to 37% in the 14 day controls; the distribution was as follows:

control (day 0) - serous 17%, transitional 2%, mucous 0%
control (day 14) - serous 17%, transitional 20%, mucous 0%
TS-exposed (day 14) - serous 18%, transitional 21%, mucous 3%

Comment: The decrease in the total labelled population observed in this experiment could be the result of cell loss from the epithelium due to either necrosis and sloughing or migration, or cell proliferation so that label dilution below the threshold number of grains which designated 'a labelled cell', occurred. The last is the most likely, despite the lack of significant increase in the concentration or percentage of labelled cells from days 0 to 10 in the concurrent proliferation studies (Experiment 2), as cell necrosis was never observed. The pattern of grain distribution change was similar in this experiment to that observed in Experiment 3; however in this experiment, changes in TS-exposed animals were of similar type and size to those in the controls, except for a small increase in the labelled ciliated cell proportion above control levels, probably due to differentiation from basal cells. TS exposure did not produce discernable change in the secretory compartment, as measured by grain re-distribution.

Experiment 5

1. The percentage of the labelled cell population that was basal decreased (from 58% to 22%) and that which was ciliated increased from 0 to 41% after 14 days of TS exposure, compared to a change over the same time in the control group from 0% to 11%. The serous percentage of the labelled population remained constant between 0 and 14 days and there were virtually no transitional or mucous cells labelled by 14 days.

Comment: As in Experiments 3 and 4, the data from this experiment suggests that differentiation, as monitored by grain loss and gain, produces more ciliated cells as a result of TS exposure. By contrast, almost no labelled secretory cells (mucous or transitional) appeared. This observation, while tentative due to the paucity of data, follows the same pattern as that from the other differentiation experiments which suggest that differentiation pathways from basal cells are the providers of the TS-induced increased ciliated cell population.

OVERALL CONCLUSIONS

1. Exposure of airway epithelium to TS produced a graded response as judged by:
   a) increases in the proportions of epithelial mucous cells and
   b) increased cell proliferation, in either secretory or basal cell compartments (Table 6.1).
   These observations can be related to the conditions of exposure (see Chapter 7.4.1).

2. In the two out of three experiments where TS produced a proliferative response, transitional and mucous cells divided in one experiment and basal cells (predominantly) and mucous cells, in the other.

TABLE 6.1

COMPARISON OF EXPERIMENTAL RESULTS

Variable	Experiment			
	5 >	1 & 3 >	2 & 4 >	Control
% epithelial mucous cells: @ 14 days	21	14	1	0.3
	5 >	1 >	2 =	C
Max. total no. labelled cells: (cells/10 HPF : proliferation)	10 (24 h)	4 (7 d)	1 (3 d)	1
	1 >	5 >	2 ≥	C
Max. no. labelled mucous cells: (cells/10 HPF : proliferation)	2 (7 d)	0.2 (14 d)	0.02 (24 h)	0
	5 >	1 >	2 =	C
Max. no. labelled basal cells: (cells/10 HPF : proliferation)	9 (24 h)	1 (7 d)	0.8 (day 0)	0.8
	3 ≈	1 >	4 >	C
Differentiation : labelled ciliated cells: % of total labelled population @ 14 days	43	41	30	16

3. The data suggest that ciliated cells differentiatied from basal cells and that TS exposure accelerated this process resulting in an increase in the size of the ciliated cell population.

4. TS exposure did not effect the differentiation pathways to secretory cells (serous, transitional and mucous cells) ie. this compartment did not increase in size due to differentiation, but the distribution of cell types within the group changed, suggesting a metaplastic response.

5. The relative differences between each experiment are illustrated in Table 6.1.

CHAPTER 7 - DISCUSSION

The studies to be discussed confirm that the population of proliferating cells in normal rat airway epithelium is small and composed of basal, secretory (serous and transitional) and indeterminate cells. They suggest there are two major pathways of differentiation:

(i) basal → (indeterminate) → ciliated

(ii) serous → (indeterminate) → transitional/mucus

The epithelium can respond to tobacco smoke (TS) exposure in a complex way which is dependent on the exposure conditions and comprises proliferation of either mucous or basal cells, alteration of the pathways of differentation to produce more ciliated cells and metaplasia of secretory cell types. It appears that TS-induced proliferation can be attenuated and delayed by concurrent administration of the mucolytic drug N-acetylcysteine.

7.1. RESPONSE OF EPITHELIUM TO IRRITATION: ROLE OF THE MUCOUS CELL

Airway epithelium has been classified by its kinetic charactaristics as an expanding system (Wright and Allison, 1984): a very small number of cell divisions occur, at a rate which correlates with the growth rate (Kauffman, 1980) and decreasing with age (Bolcuc and Reid, 1976) and the tissue has the capacity to recruit cells into proliferation when necessary, as during response to injury.

7.1.1. Epithelial Stem Cells

The results of the experiments reported here confirm that the major proliferating population of SPF rat airway epithelial cells consists of basal (71% of the labelled population), serous (17%) and indeterminate cells (9%). During irritation with TS, the proliferative response of each epithelial cell type was monitored, demonstrating that under different conditions, either primarily mucous cells, or primarily basal cells responded to the irritative stimulus. These findings focus attention upon an issue which is currently being re-evaluated, namely, which cells are stem cells in mammalian airway epithelium?

Stem cells are defined as a self-replicating cell group, which may or may not be functional and give rise to other cell types. Historically, this label has been applied to basal cells (Drasch, 1881; Blenkinsopp, 1967; Bindreiter, 1967, Breeze and Wheeldon, 1977; Kauffman, 1980), due to their basal position in the epithelium (reminiscent of basal cells in skin), because they appear to be unspecialized compared to secretory and ciliated cells and because, at least in rat extra-pulmonary airway, a larger proportion of this compartment than any other, is normally found to be proliferating (Wells, 1970; Bolduc, 1976). However when the epithelial proportions and proliferative activity of basal and secretory cells are examined and compared, variation between species and airway levels within the same species becomes apparent.

Basal cells are generally frequent in upper airways of mammals

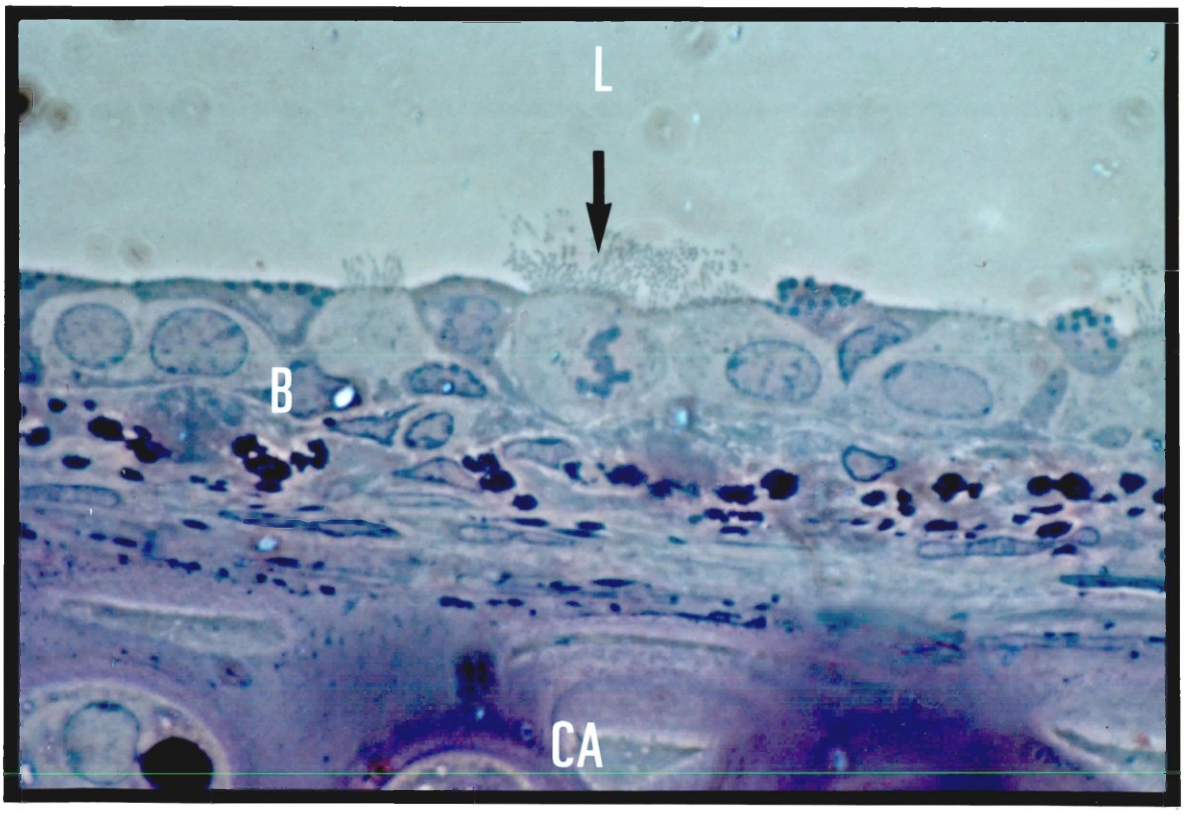
but are rare or absent in peripheral airways, whereas secretory cells are present at all levels (Jeffery and Reid, 1975; Castleman et al., 1980; Pack et al., 1981). Both basal and superficial dividing cells have been identified in a variety of animals (Spencer and Shorter, 1962; Divertie et al., 1964, 1966; Blenkinsopp, 1967; Wells, 1970; Harris, 1975; Bolduc and Reid, 1976), but few quantitative studies have compared the relative contributions of each epithelial cell type to the proliferating population. Those that have, in rat and hamster only (Boren and Paradise, 1978; Donnelly et al., 1982; Keenan et al., 1982 a-c; 1983; Evans et al., 1986) show that while both basal and secretory cells normally proliferate, in rat proliferating basal cells are in the majority, but in hamster secretory cells outnumber basal cells in their contribution to the proliferating population.

In bronchioles and alveoli, where basal cells are rare or non-existant, control studies for NO_2 and O_3 exposures have demonstrated that non-ciliated or Clara cells and type II alveolar cells comprise the resting proliferating population in these tissues ie. 1h after ^{3}H-T administration to uninjured bronhiolar epithelium, 80.5% of the label is in non-ciliated cells without granules or smooth endoplasmic reticulum (SER) (ie. type A cells), 13.5% in nonciliated cells with granules but no SER (ie. type B cells) and 6.5% in Clara cells, nonciliated cells with both granules and SER (Evans et al., 1978a). Type A and B cells were regarded by the authors as 'intermediate' cells, ie. potential Clara cells. No other epithelial cells became labelled. Likewise, in control alveoli 89% of the labelled population were type II cells and no type I cells took up label (Evans et al., 1976a).

What of other members of the epithelial population? The data reported herein indicate that 9% of the dividing population in SPF rat main bronchial epithelium are indeterminate and 2% ciliated cells. Indeterminate cells are a composite group, as demonstrated in hamster by McDowell et al., (1983), most probably composed of longitudinally orientated basal cells and serous cells which have discharged their granules as well as a small number of genuine indeterminate cells. It is thus difficult to be certain of the real contribution of this group to the proliferating population without means of precisely identifying its components. Ciliated cells are generally regarded as terminally differentiated end-cells which do not divide (Boren and Paradise, 1978; McDowell and Trump, 1983). However labelled ciliated cells were sometimes, albeit rarely, observed in the experiments discussed herein (Tables 3.3, 4.4 and A.1.1-A.1.1.3). Evans et al., (1976a), by comparing labelled ciliated cell counts of 1μ plastic sections done with light microscopy, with counts of the same tissue using electron microscopy, showed that some ciliated cells had been mistakenly identified using the former technique. This may be the explanation for the low positive labelled ciliated cell score obtained here. However, ciliated cells have been shown to divide in response to isoproteronol (IPN) (Jeffery, 1973) and as shown in Fig. 7.1, ciliated cells, rarely, do appear to be in mitosis. In any experiment this question can only be resolved by electron microscopic studies.

Thus, accumulating evidence, supported by the observations reported herein, indicates that the stem cell population which

FIGURE 7.1 Control epithelium: rat main bronchus. 1μ section stained with Toluidine Blue showing what appears to be a Ciliated cell in mitosis (arrow). Basal cells (B), Lumen (L), Cartilage (CA).
(X 1300, Phase optics)


L
B
CA

contributes to maintainance in normal healthy epithelium, ie. those cells observed to be proliferating in the resting tissue, are secretory as well as basal cells: serous and mucous cells in proximal airways, Clara and type II alveolar cells in distal airways and alveoli.

The question of whether airway epithelial proliferation manifests circadian variation was addressed in one experiment herein. In previous studies conflicting findings have come from 6 reports: 3 studies using rats have observed a morning peak in labelling index (Messier and LeBlond, 1960; Romonova, 1966; Sokolova, 1975) and 3 studies of mouse and hamster found a proliferation peak in the evening (Koburg, 1962; Kmoch, et al., 1970; Das et al., 1979). This variation could reflect species differences, or just differences in technique. The fact that this small experiment demonstrated a morning peak in labelling due only to serous cells is interesting, especially as it seems to fit with the other studies on rat, but clearly no solid conclusions can be drawn and detailed work examining changes in proliferation rates sequentially over 24 hours is needed.

7.2.2. Role of the Mucous Cell in Response to Irritation: Changing Concepts

As is indicated from data reported in Chapters 4 and 5, basal cells and importantly, mucous cells, proliferate in response to an irritative stimulus in SPF rat extrapulmonary airway epithelium. These observations comfirm and extend previously published studies of a variety of models of injury, including exposure to toxic gases

eg. NO_2, O_2, SO_2 and O_2 and different degrees of mechanical injury. These stimuli invoke a common response, which can be compared with that observed herein in response to TS. A wave of proliferation is induced by injury, the size and duration of which is proportional to its' severity. (Wilhelm, 1952; McDowell et al., 1979, Shami et al., 1982). Restoration of a functional mucociliary epithelium occurs by differentiation into mature secretory and ciliated cells (Wilhelm, 1952; McDowell, 1979; Keenan et al., 1982a-c; 1983; Shami et al., 1982). The details of this process are still not clear and controversy about the cell or cells which have reparative capacity continues.

Comparison of studies of toxic gas exposure are rendered difficult by the great variation in species, dose, duration and manner of administration. In addition, very few studies, particularly those of changes in the extrapulmonary airways, compare and quantitate the proliferative response of each cell type. Nevertheless, results of these studies do have features in common: firstly, a relationship between dose of irritant administered and the amount of cell proliferation produced; secondly, both basal and mucous cells have been reported as the responsive proliferating cell. Exposure to SO_2 (Lamb and Reid, 1968), O_3 (Penha and Wertheim, 1974) and TS (Bolduc and Reid, 1981; Jeffery and Reid, 1981) induced proliferation of tracheal basal cells (these studies were all carried out in rats). In a recent study of O_3 exposure in monkeys (Wilson et al., 1984) basal and small mucous granule (SMG) cells in the trachea proliferated and gave rise to 'intermediate' cells with both mucous and ciliated characteristics. NO_2 exposure induced proliferation of secretory cells, but not basal cells in rat

proximal intrapulmonary airways (Evans et al., 1986). The studies of Wilson and Evans and their colleagues are two of very few quantitative morphological and autoradiographic studies which use light microscopy of plastic-embedded sections and correlative electron microscopy. These techniques enable the precise identification of cell types which is necessary if the response of the whole epithelium to irritation is to be effectively monitored and reliable information about stem cells gained.

New insights into the relative roles of mucous and basal cells have come from detailed studies of repair after mechanical injury. Early experiments by Condon, (1942) and Wilhelm, (1952), conducted in rat trachea, concluded that the basal cell was the active initiator of the repair process. Gordon and Lane (1974, 1977, 1980) described two separate epithelial populations also in rat tracheal epithelium, which, normally at rest in the G_1 and G_2 phases of the cell cycle, responded to mild injury with one passage through the cell cycle; the populations were identified as basal and mucous cells (Lane and Gordon, 1979).

Keenan et al., (1982a-c, 1983), studied repair of mechanical injury in hamster trachea and by detailed morphological and autoradiographic observations, in particular very sensitive detection of intra-cellular mucous secretory material, present interesting evidence supporting the primary role of mucous cells in this process. They found that in control hamster epithelium, the secretory to basal cell ratio is 6:1; immediately after wounding, the cells which flattened and covered the deficit were mainly secretory. This population began proliferating, at an increased rate

and 80% of these dividing cells were secretory. The resultant daughter cells produced an epidermoid metaplastic epithelium and many cells of this population contained both keratin and secretory granules. As proliferation continued, albeit at a reduced rate, immature columnar cells, designated by the authors pre-ciliated and pre-secretory, were morphologically identifiable by the presence of basal bodies and secretory granules; these cells each differentiated into their respective mature forms. The authors demonstrated convincingly that these immature cells had arisen from dividing secretory cells and compared them with similar cells observed in the epithelium of developing hamster (McDowell and Trump, 1983) and in human airway epithelium (Becci et al.,1979).

In the extensive review paper by McDowell and Trump (1983) which draws together all this data, the hypothesis is advanced that "secretory cells play a major role during fetal development, normal adult epithelial turnover and during regeneration" and the prediction made that this phenomenon is common to many species. Further evidence involving comparison between epithelial response to experimental carcinogen exposure and studies of human neoplastic lesions suggest to the authors that the mucous cell is also instrumental in the development of the epidermoid metaplasia which is prominent in both of these pathological situations and gives rise to carcinoma-in-situ.

In the face of this impressive array of evidence, the basal cell is being pushed to retire from its former prominent place as the airway epithelial 'stem cell' by the mucous cell. In the view of McDowell and Trump, (1983), the large tono-filament content of basal

cells indicates that they are a specialized type which may have a role, as yet little understood, in the maintenance of epithelial structural integrity. However the observation in the present work of a large basal cell proliferative response to high tar cigarettes, the findings of Gordon and Lane, (1980), of basal cells as well as mucous cells entering the cell cycle in response to injury, the report by Wilson et al., (1984), of basal and superficially placed cells proliferating in response to O_3 all demonstrate the proliferative and reparative capacity of basal cells. Taken together, this evidence suggests that in airway epithelium two stem cells, mucous and basal, operate of which the mucous cell is the more important.

These experimental findings regarding the mucous cell are important in the light of clinicopathological studies which strongly suggest that the aberrant presence of mucous cells in distal airways may be an early pathological indicator of potential development of destructive lesions and contributes to the clinical problems produced by advanced disease (Hogg et al., 1968; Matsuba and Thurlbeck, 1982; Thurlbeck, 1982; Lumsden et al., 1984) (see Section 7.5).

7.2. PATHWAYS OF DIFFERENTIATION

The aim of the experiments described herein was to examine the effect of TS on pathways of differentiation by observation over the experimental time period, of the change in distribution of label within a cohort of cells, labelled with ^{3}H-T once only on day 0.

These data encompass changes due both to differentiation, usually regarded as an irreversible process in which a non-specialized cell becomes a functional mature cell while passing through one or more kinetic compartments, and metaplasia, a change from one functional cell type to another which may or may not be reversible (Scarpelli, 1985).

The results of each experiment show that in both control and TS-exposed groups, the basal cell compartment, which originally contained the highest proportion of labelled cells, decreased its contribution to the labelled population with time; this process appeared to be accelerated in TS-exposed animals. Concurrently, the percentage of the ciliated compartment which was labelled increased, to a greater extent in TS-exposed than control animals. Although the contribution of the secretory compartment to the total labelled population in both Experiments 3 and 4 increased with time, at 14 days the difference between TS-exposed and control animals lay in the distribution of cells within this compartment, rather than its size.

These data indicate that in this system basal cells gave rise to ciliated cells, probably via indeterminate cells. TS appeared to increase production of ciliated, but not secretory cells, by differentiation pathways and induce transitional and mucous cell production due to metaplasia of serous cells.

Metaplastic change in response to TS has previously been reported by Jeffery and Reid (1981) who observed the appearance of

'transitional' cells ie. cells containing both dense and lucent granules and suggested that rat airway serous cells changed their secretory product giving rise to mucous cells. In response to SO_2 exposure, Lamb and Reid, (1968), showed that in rat distal airways, mucous cells appeared and increased in number, without significant increase in the epithelial mitotic index, suggesting a metaplastic change. These observations demonstrate that airway epithelial secretory cells are capable of changing their product in response to environmental stimuli, a change which the findings of Rogers and Jeffery, (1986c), indicate are reversible upon removal of the irritative stimulus.

Studies of differentiation in unirritated airway epithelium present apparently conflicting findings. The role of the intermediate (indeterminate) cell was highlighted by Donnelly et al., (1982), who used changes in the distribution of adjacent labelled cell pairs (representing recent daughter cells) with time, as an indicator of differentiation in normal rat airway epithelium. Their observations lead to the conclusion that both ciliated and mucous cells arose from dividing indeterminate cells which in turn resulted from basal cell divisions. However, as McDowell et al., (1983), pointed out, this cell group, at least in hamsters, is a composite of several components, thus assessment of its real contribution to airway renewal is problematic. Boron and Paradise, (1978) proposed a three compartment model for hamster tracheal epithelium, comprising stem cells (basal cells) which replaced themselves and gave rise to a maturation or differentiation compartment, containing mucous cells, some of which retained the ability to divide. The third compartment contained only fully

differentiated cells which did not divide, ie. ciliated cells. Their findings provided strong evidence that mucous cells differentiated into ciliated cells.

Increasingly, recent studies of disturbance to airway epithelium indicate that the epithelial population is restored after injury by differentiation from daughters of mucous cells which have proliferated in response to a stimulus. Gordon and Lane (1984), in a study in which the superficial layer of rat tracheal epithelium was stripped by mild mechanical means, observed that the remaining cells cycled once. These cells were made up of two groups, basal cells at rest in G_1, and superficial mucous cells in G_2. Change in the percentage distribution of label in the cycling populations with time and grain counting, showed that basal cells almost invariably gave rise to basal cells only, whereas superficial cells gave rise to more secretory cells and subsequently ciliated cells. The recent study of Evans et al., (1986), also in rats, showed that on exposure to NO_2 the combined secretory compartment (cells containing dense secretory granules, indeterminate cells, pre-secretory cells and mucous cells) of the tracheal epithelium proliferated, then differentiated into a new population of ciliated and secretory cells; basal cells did not respond to the irritative stimulus.

McDowell et al., (1987b), in a study of growth and differentiation of hamster mucociliary epithelium in primary culture observed that a mono-layer of poorly differentiated flattened cells, mostly altered secretory cells, developed into cuboidal epithelium containing apical mucous granules by days 4 and 5, preciliated and newly formed ciliated cells by day 6 and a differentiated

mucociliary epithelium on day 7. This developmental time scale *in vitro*, was compared to time scales observed *in vivo*, during regeneration after injury (Keenan et al., 1982a, b, c; 1983), vitamin A deficiency and restoration (McDowell et al., 1984 a and b) and development of hamster tracheal epithelium (McDowell et al., 1985; Otani et al., 1985); the time taken for production of a mature muco-ciliary structure was equivalent in each experimental situation. These authors have thus contributed yet more evidence in support of their thesis that the mucous cell is the primary stem cell of airway epithelium, providing pre-secretory and pre-ciliated cells, which then differentiate into the mature cell forms.

Thus the balance of accumulating evidence from studies of injury and repair points to the mucous cell as the most important airway epithelial stem cell. However, the observations of differentiation pathways in normal and TS-exposed rat main bronchial epithelium reported herein indicate that basal cells differentiate (probably via indeterminate cells) to form ciliated cells, a process accelerated by irritation, which also induced increased proliferation in both mucous and basal cell compartments; these findings are comparable to those of Donnelly et al., (1982). Clearly much work remains to be done towards the clarification of this question; improvements in the methods used herein to enable gathering of more precise information about these processes, have been outlined in Sections 7.4 and 7.6.

7.3. MODULATION OF TS-INDUCED RESPONSE BY DRUGS

The studies reported here have shown that the TS-induced proliferative response in epithelium of SPF rat main bronchus can be delayed, decreased and attenuated by the concurrent administration of N-acetylcysteine (Nac). In addition, the increase in mucous cell numbers observed at 14 days in the TS-exposed group was decreased significantly in animals given Nac. These findings are supported and complimented by Rogers and Jeffery (1986a), who found that Nac inhibited the TS-induced increase in acidic glycoprotein-containing mucous cells, but did not prevent a decrease of cells secreting neutral glycoprotein.

This is the first demonstration of modulation by a mucolytic drug of mucous cell numbers and epithelial proliferation and differentiation, although several other drugs have previously been shown to change the proportions of airway epithelial mucous cells. Of these, the neuromimetics isoprenaline (IPN), salbutamol (SAL) and pilocarpine (PCP), administered intraperitoneally, increased the mucous cell numbers (Bolduc and Reid, 1978; Jones and Reid, 1979; Jeffery et al., 1983b). Concurrently, epithelial proliferation was increased by IPN and PCP and notably, more superficial than basal cells were observed in mitosis (Bolduc and Reid, 1978). In addition, the mucous cell product was modified towards acid glycoprotein, whether cell number was increased or not. In salivary gland, IPN activates adenyl cyclase and causes an increase in cyclic-AMP levels at the same time as increasing mitosis (Malamud, 1969; Durham et

al., 1974). As discussed in Chapter 1.1.4., cyclic-AMP levels are thought to be closely related to the balance between proliferation and differentiation among a variety of tissues. The report of Nygren et al., (1984), of increased mucous cell numbers after administration of di-butryl cyclic-AMP to rats, tends to support the suggestion that the IPN-induced increase in mucous cells in airway epithelium occurs via this mechanism.

Cyclic-AMP also plays a role in mucus synthesis and secretion: in vitro, it increases the incorporation of radio-active precursors of mucus into glycoprotein molecules and causes mucous secretion (Goggins et al., 1972; Forstner et al., 1973; Whimster and Reid, 1973). These findings make it likely that IPN at least, and perhaps also PNP and SAL, influence airway epithelia by a combined effect on proliferation and mucus synthesis and secretion.

Inhibition of mucous cell increase by drugs has also been investigated. Jones et al., (1972, 1973), have shown that TS-induced mucous cell increase was inhibited by the non-steroidal anti-inflammatory drug phenylmethyloxadiazol (PMO), (administered in TS, 2% by weight), in extra-pulmonary but not intra-pulmonary airways. At 24h and 7 days a TS-induced proliferative response was observed, which PMO decreased in amount and delayed in time.

Studies of the effects of the non-steroidal antiinflammatary drugs indomethacin and flurbiprofen and the steroids dexamethasone, prednisolone and hydrocortisone (administered at a dose of 4mg/kg intraperitoneally) on TS-induced mucous cell increase (Grieg et al. 1980; Rogers, 1984; Rogers and Jeffery, 1986b) found that each of

these substances inhibited the increase, in both extra and intra-pulmonary airways, to a varying extent. The efficacy of inhibition varied in the same order as that associated with classical inhibition of inflammatory reactions

ie. flurbiprofen < indomethacin

and dexamethasone < prednisolone < hydrocortisone

(Rogers, 1984b). Indomethacin, dexamethasone and hydrocortisone have been shown to inhibit cell proliferation in a wide variety of systems eg. human fibroblasts and rat hepatoma cells (Bayer et al., 1979), 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated rat and mouse epidermis (Francis and Marks, 1979), regenerating liver (MacManus and Braceland, 1976), hormone-stimulated pituitary and thyroid glands (Sluszkiewicz and Pawlikowski, 1980; Pawlikowski et al., 1981) and TPA-promoted, DMBA-induced tumours in vivo (Belman and Troll, 1972; Weeks et al., 1979). Within these systems, the potency of the inhibitory action by steroids was again in the same descending order as the power of their anti-inflammatory actions (Belman and Troll, 1972).

It has been suggested that the drug-induced mucous cell decrease resulted from a decreased rate of epithelial cell proliferation, mediated via decreased synthesis of the arachadonic acid metabolites (Greig et al., 1980; Rogers, 1984; Rogers and Jeffery, 1986b). These substances, particularly the prostaglandins, have been shown to increase cyclic-AMP levels, and thereby stimulate cell proliferation, in many tissues (Robinson et al., 1971; Molnar et al., 1987) and indomethacin is a well established prostaglandin synthetase inhibitor.

In contrast to indomethacin, flurbiprofen and the steroids, N-acetylcysteine (Nac) has been used for some years as an orally administered mucolytic for the treatment of chronic bronchitis, but despite numerous and continuing clinical trials its efficacy has remained controversial (Wanner and Rao, 1980; Richardson, 1987). Recent studies in patients with chronic bronchitis have shown both no significant change in the number of acute exacerbations (British Thoracic Society, 1985; Parr and Huitson, 1987) and a significant decrease in acute episodes of exacerbation (Boman et al., 1983); no beneficial effect on mucociliary clearance (Millar et al., 1985) and an increase in the rate of 'slow' mucociliary-clearance (Todisco et al., 1985); and in addition, no effect on spirometric values or pulmonary symptoms, including mucus production, in patients with asthma (Bylin et al., 1987).

The earlier assumption based on in vitro studies (Sheffner et al., 1963, 1964), that Nac decreases mucous viscosity directly by breaking disulphide bonds between mucous glycoproteins, has been challenged by Millar et al., (1985) and by a recent report that oral Nac, given daily for two weeks to healthy volunteers, was undetectable in either plasma or bronchial lavage fluid. Interestingly, however, the plasma glutathione levels of the Nac recipients in this study were raised (Cotgreave et al., 1987).

It is well established that all cells contain enzyme systems which protect against damage by the highly reactive free radicals (superoxide ion, $O_2^{\cdot-}$, hydrogen peroxide, H_2O_2, hydroxl radical, $^{\cdot}OH$ and singlet oxygen, $O^{\cdot}$) generated by oxidation-reduction reactions during cellular metabolic processes (Clark, 1986). These

intra-cytoplasmic enzymes include glutathione peroxidase, glutathione S-transferase and glutathione reductase, which catalyse reactions of the intra-cellular tripeptide thiol glutathione (GSH) with potentially damaging electro-philic compounds and free radicals (Meister, 1983). These enzymes have been identified in both rat and human lung although the levels vary across species (Jenkinson et al., 1984). The importance of GSH was demonstrated when depletion of GSH levels by nitrosurea resulted in a 30 fold increase in the susceptibility of type II alveolar epithelial cells to polymorphonuclear neutrophil (PMN)-mediated cytotoxic damage (Simon, 1986).

As well as being used clinically as a mucolytic, Nac has been shown in several systems to be protective against free radical generated damage. It is currently the antidote of choice against massive paracetamol-induced liver damage in humans (Prescott, 1979; Prescott, 1982), and has been shown to protect against hepato-toxicity of acetaminophen in vitro by increasing synthesis of glutathione, thus providing more substrate for detoxification (Lauterburg et al., 1983; Harmen and Self, 1986).

By scavenging the products of the degradative enzyme system myeloperoxidase (isolated from human polymorphs), Nac prevented inactivation of the α1-proteinase inhibitor. (Borregaard et al., 1987). Lung epithelial cells were protected by Nac in vitro, from PMN derived O_2 metabolite-induced damage (Simon and Suttorp, 1985). TS-induced inhibition of alveolar macrophage phagocytic ability in vitro was abolished by addition of cysteine (a metabolic product of Nac), or glutathione to the system in a dose-response related way

(Green, 1968).

Interestingly, Leuchtenberger and Leuchtenberger 1977, report that in cultures of lung cells (which were unidentified), L-cysteine decreases the TS-induced abnormalities in cell division and DNA content and the increased growth rate shown by the fibroblasts in the culture. Unfortunately, there was no mention of the nature of the cell division abnormalities nor were they quantified, making comparison with other studies difficult.

A much later report showed that as a result of exposure of alveolar type II cells to O_3, reduced and oxidized GSH levels decreased (Rietjens et al., 1985). It is interesting to examine this report along with the work by Evans and collegues who studied the morphological effects of O_3 exposure and found a dose-dependent, short-lived wave of cell proliferation in Clara cells and type II alveolar epithelium (Evans et al., 1971, 1976a and 1976c). In the light of these observations, it would be of interest to know whether Nac also inhibited O_3 or NO_2-induced proliferation and to measure GSH levels in extra-pulmonary airway epithelial cells exposed to TS.

These observations strongly suggest that the protective functions of Nac are mediated by direct combination with cytotoxic products of cell reactions and by increasing the synthesis of glutathione, and its enzymes.

In its gas phase, TS contains a high concentration of oxidizing agents eg. a variety of nitrous oxides, acrolein, formaldehyde (US Surgeons-General's Report, 1979) which generate

free radicals. There is increasing evidence from studies of tumour promotion, of which hyperplasia is a major componant (Baird et al., 1971; Slaga et al., 1976), that free radicals themselves stimulate cell proliferation. It has been demonstrated that small quantities (5-10 ng) of the tumour promotor phorbol-myristate-acetate (PMA) are enough to stimulate the production of superoxide ion (O_2^{-}), and H_2O_2 in PMN's. The degree of stimulation of free radical production correlates with the promotion activity of PMA and other promoting substances in mouse epidermis (Troll and Weisner, 1985). In guinea pig macrophages, other phorbol esters which are inactive as tumour promotors do not produce O_2^{-} (Goldstein et al., 1981). FR generating compounds eg. benzoyl peroxide and chloroperbenzoic acid are effective skin tumour promotors in mouse epidermis after initiation with di-methyl-benzanthracine (DMBA) (Slaga et al., 1981). Generation of free radicals in mouse epidermis by TPA has been measured by chemiluminescence, an index both of generation of and mediation of reactions by free radicals (Fischer and Adams, 1984).

It is of great interest that the steroids dexamethasone, prednisolone and hydrocortisone, inhibit tumour promotion (Belman and Troll, 1971) and that indomethacin inhibits the chemiluminescence induced by TPA in mouse epidermis (Fischer and Adams, 1984). These observations suggest a link between free radical production and prostaglandin levels. It would be interesting to compare effects of Indomethacin and Nac and possible synergism between them, on TS-induced cell proliferation.

There remain many unanswered questions. What is the nature of the apparent difference in the effect of Nac on mucous and basal

cells? Are there different levels of glutathione in mucous and basal cells? Does Nac have its effect intra- or extra- cellularly, directly or indirectly? These questions deserve further investigation.

Combination of the above findings with reports of the protective effects of Nac against cell damage induced by free radicals, strongly suggests that Nac may be mediating its effect on TS-induced cell proliferation by protecting glutathione/glutathione enzyme system against breakdown, and/or stimulating glutathione synthesis; both of these mechanisms could prevent cell damage by free radicals and thus prevent subsequent cell proliferation.

7.4. LIMITATIONS

The factors in this study which impose limitations upon the interpretation of the results will now be discussed.

7.4.1. The Animal Model

The experiments discussed herein were carried out using a model which has provided considerable information about epithelial response to irritation and its inhibition (Lamb and Reid, 1969; Jones et al., 1972; Jones and Reid, 1978; Jeffery and Reid, 1981; Rogers and Jeffery, 1986b). With time the basic structure of the model has remained the same ie. specific pathogen free (SPF) rats exposed to 25 cigarettes over 4 hours, daily, for a minimum of 14 days and a maximum of 6 weeks. Its major features have been a dose-response relationship

between the number of cigarettes smoked and the amount of mucous cell hyperplasia and cell proliferation (Lamb and Reid, 1969), and a histochemical shift in the type of intracellular mucin produced from neutral to acid glycoprotein (Jones et al., 1973). Morphologically, the shift was represented by both an increase in secretory cells producing acidic glycoprotein and a decrease in those producing the neutral variety. In more recent studies using this model (Rogers, 1984), the dose-response relationship was observed and expressed as a quantitative relationship between secretory cell hyperplasia and the combined cigarette tar level and TS exhaust rate from the exposure cabinets. However, the histochemical shift was infrequent in this study, observed by Rogers in one experiment only. It was suggested that the change was due to a decline in cigarette tar levels over recent years (Laboratory of the Government Chemist, 1973-1983). Further assessment of the experimental results lead to the conclusion that the amount of hyperplasia was proportional to the amount of TS to which the rats were exposed, but the presence or absence of the histochemical shift was determined by the 'quality', ie. tar and nicotene levels, of the smoke.

These observations are interesting in the light of the findings reported herein, in which different, but related epithelial responses were obtained in three experiments using this model of TS exposure. The 3 experiments have in common that, in response to TS, numbers of mucous secretory cells increased, serous secretory cells decreased, differentiation pathways appeared to produce more ciliated cells and control animals showed similar, though significantly smaller, changes of mucous and serous cell numbers to those induced by TS. Differences lie in the size of the mucous cell increase, the size and time scale

of the proliferative response and the cell type which proliferated (see Table 6.1). The size of the response was by far the greatest in Experiment 5, carried out after the cigarettes were changed to those with medium to high tar levels; this experiment was done concurrently with the one in which Rogers, (1984) observed a histochemical shift in mucus type. There is also a clear difference between Experiments 1 & 2 in terms of the increase in the mucous cell proportion of the airway epithelium and the proliferative response, suggesting a higher level of TS exposure in the former, despite use of the same cigarette type in both. These experiments were separated by 20 months and it is possible that the cigarette tar levels decreased during that time sufficient to cause this difference. In addition, during this time a new set of smoking machines came into operation. They were constructed to standard specifications and no comparison was made between old and new machines of the TS concentration generated inside the cabinets. It is therefore likely that their use resulted in a lower intra-cabinet level of TS.

It is clear from comparison of changes in cell concentrations in both control and TS-exposed groups in all experiments (see particularly Figs. 4.4. - 4.6, Table 5.1), that changes, of similar type but lesser degree, occur in control animals, especially in the mucous and serous cell compartments. The observations suggest that airway epithelium responds rapidly to less than 'barrier maintained' conditions, thus protection of controls from 'contaminated' atmosphere where possible and more important, careful monitoring of controls by paired sampling from control and exposed groups at each time point is highly desirable.

These observations illustrate the ability of the epithelium to mount a response appropriate to the stimulus, but also the great importance of control and monitoring of the parameters of the exposure system particularly the cigarette tar and nicotene levels and the intra-cabinet concentration of TS. Substantial steps have been taken in that direction by Rogers, (1984) as already described; additional correlations were made in his study between mucous cell hyperplasia and CO exposure and plasma concentrations of COHb and nicotene (Rogers and Jeffery, 1986a).

7.4.2. Techniques and Interpretation

In these studies, Labelling Index (LI) was used as a measure of cell proliferation and differentiation instead of mitotic index (MI), because the time of the DNA synthesis (S) phase relative to the time taken for mitosis is longer (Aherne et al., 1977), thus a larger number of cells can be targeted and labelled by ^{3}H-T. However, as ^{3}H-T is available for approximately 30 minutes, (Feinendegen, 1967), pulse labelling captures only a small proportion of the small number of proliferating cells in airway epithelium. This approach necessitates the counting of very large cell numbers to gather a statistically viable amount of data, which in these experiments amounted to 2600 - 6000 cells per animal. As outlined in Section 7.6., the use of the thymidine analogue bromodeoxyuridine (BUdR), appropriate monoclonal antibodies and the Fluorescence Activated Cell Sorter (FACS), would enable a more accurate and efficient determination of LI, the proportions of each cell population in each phase of the cell cycle and the changes in these populations and proportions due to injury.

Changes in the number of cells labelled by a single pulse of ^{3}H-T were used in two ways in these experiments: to measure the number of cells proliferating in each epithelial compartment 1 h after administration at each time point during TS exposure; and to measure differentiation by assessment of change in percentage label distribution throughout the epithelial cell compartments during the 14 day experimental period. Cells were visualized with autoradiographic techniques and the assumption made that a cell was labelled when overlaid with 4 or more grains. The labelled cells observed, were for the most part, overlaid by many more than 4 grains, with the number of grains per unit area much higher than that found in either control sections without ^{3}H-T, subjected to identical autoradiographic procedures, or in areas of the embedding plastic where there was no tissue. However the qualitative nature of this designation of when a cell is 'labelled' is clearly not sufficient, particularly in the experiments aiming to monitor differentiation. The data from the latter experiments was made less precise by the lack of quantitative determination both of the threshold grain number designating 'a labelled cell' and of the level of background: thus it is possible that proliferation in any one compartment could have lead to 'drop-out' of cells from the originally labelled group due to dilution of grain number below the fixed 'threshold' value. A useful quantitative means of more accurately determining when a cell is labelled is mean interphase grain counting of samples of unlabelled and labelled cells from proliferating compartments at each time point for control and treated groups: a well defined peak in grain number should be associated with cells which were labelled (Wright and Allison, 1984).

An area where the differentiation data could be improved is in the gathering of data regarding ongoing proliferation in the originally labelled cell cohort, to enable accurate identification of the cell products of differentiation and metaplasia. Comparison of mean grain counts over the labelled cells in each compartment with time would enable the labelled population to be classified into three groups, cells which are non-proliferative, those which proliferate once and those which proliferate twice (Baserga et al., 1963; Boren and Paradise, 1978). This procedure would monitor the proliferation occurring and allow a clearer understanding of the derivation of functional epithelial cell types and changes induced by TS exposure.

As can be seen from Chapter 6, there are 3 sets of experiments presented herein, in which TS-induced changes in airway epithelial proliferation and differentiation were observed. In the first experimental set (Experiments 1&3) there are no day 0 control animals against which to compare TS-induced changes. It was therefore decided to use the control day 0 data from Experiments 2&4 and Experiment 5 for comparison; both the latter data sets are within the same quantitative range suggesting that there is little variation in size and cell types in the proliferating population of main bronchial epithelium from normal SPF rats of the same species and age.

7.4.3. Analysis

Despite the relatively small counting errors incurred in compilation of the quantitative data, (see Chapter 2.7), it is clear from the high standard errors for labelled cell concentrations, that there is considerable variation present in these populations.

Preliminary work done developing a new method of statistical analysis for this data (see Section 6.7; Laslet, personal communication; Baker and Nelder, 1978) shows that considerable inter-animal variation in epithelial labelling frequency occurred. This finding is complimentary to the observation made during the analysis used herein, that the total labelled cell and labelled mucous cell populations followed multiple poisson distributions. This method of analysis, which combines data from all animals at each time point, using as a unit n = one section rather than n = one animal, does not enable enough account to be taken of inter-animal differences thus producing large standard errors for the infrequent events ie. cell labelling, and very small standard errors for frequent events eg. the occurrence of basal cells in the epithelial population. As discussed briefly in Section 7.6, an alternative analytical method is available which generates a heirarchy of mathematical models to describe the data and can overcome this problem.

7.5. CLINICAL RELEVANCE

Results of the work reported herein demonstrate that upon irritation with TS, airway epithelium exhibits a complex response appropriate to the strength of the stimulus, comprising proliferation, metaplasia and accelerated differentiation. The response of the secretory cell compartment is highly important, contributing both increased proliferation of transitional and mucous cells and metaplasia from serous to mucous cells.

This work was based on the animal model developed by Lamb and

Reid (1969) to study mucous cell hyperplasia, as a result of earlier studies in which Reid, (1954) demonstrated the importance of hypertrophy and hyperplasia of mucous-secreting glands and goblet cells in chronic bronchitis and emphasized the appearance of goblet cells in terminal airways, where they are normally rare. Mucous cell hyperplasia and metaplasia and hypersecretion have subsequently been reported in the early and late stages of asthma (de Haller and Reid, 1964; Cutz et al., 1978; Lopez-Vidriero and Reid, 1983) and in cystic fibrosis (Esterley, 1968).

Recent epidemiological studies seeking to correlate airflow obstruction with sputum production and progressive changes in lung function as measured by changes in forced expiratory volume in one second (FEV_1) (Fletcher and Peto, 1977), have found that among subjects with similar initial airflow obstruction, age-specific death rates from chronic obstructive pulmonary disease were not significantly related to the initial mucous hypersecretion, supporting the authors contention that "airflow obstruction and mucus hypersecretion are largely independent disease processes" (Fletcher and Peto, 1977; Peto et al., 1983).

Measurements of sputum volume and FEV_1 values, however, relate only to lesions in the first six generations of airways, but it is in the small airways (<3 mm) where hypersecretion, although contributing little to sputum volume, may have important consequences with respect to airflow obstruction (Wright et al., 1984). The site of the lesion in chronic obstructive lung disease has been localised to small airways: resistance at this site was increased 4-40 times in patients with obstructive disease, due to mucous plugging, fibrosis, narrowing

and obliteration of the small airways (Hogg et al., 1968). Subsequently, mucous cell metaplasia has been observed in patients with both chronic bronchitis and emphysema (Thurlbeck et al., 1975).

There is increasing evidence that inflammatory lesions in small airways, to which mucous cell metaplasia and hyperplasia is an important response, occur in smokers before any clincial evidence of obstruction is observed. Matsuba and Thurlbeck, (1973) found a 3-4 fold increase in lumenal mucus and narrowing of peripheral airways in patients with chronic hypersecretion (usually associated with an increase in the amount of mucous-secreting tissue in the central airways) who were smokers more than 50 years of age, but showed no evidence of chronic airflow limitation or emphysema.

In a study of lungs from healthy smokers less than 40 years old, no detectable abnormalities in central cartilagenous airways were found (Sobonya et al., 1972) yet in peripheral airways significant inflammatory lesions characterized by clusters of pigmented alveolar macrophages, mural inflammatory cells and denuded epithelium in the membranous (respiratory) bronchioles were observed; a few bronchioles contained mucus although in this study no mucous cell hyperplasia was observed (Niewoehner et al., 1974). Further studies of smokers without clinically discernable airflow obstruction, have found that goblet (mucous) cells replaced Clara cells in the terminal bronchioles (Ebert and Terracio, 1975); Ebert and Hanks, 1981, Wright et al., 1983; Lumsden et al., 1984).

The combination of these clinicopathological studies with the accumulating experimental evidence demonstrating the important role of

mucous cells in epithelial repair processes, (see Section 7.1) presages exciting movement towards greater understanding of airway epithelium, which while vital in host defence, is also instrumental in the development of common and destructive disease.

7.6. FUTURE DIRECTIONS

The present study and the resulting discussion suggest several future lines of investigation.

1. **The influence of tar content and TS exposure levels on the proliferation of distinct cell types:** the results reported here and those of other workers, suggest that epithelial proliferative responses vary according to the tar concentration to which the animals are exposed. Investigation of this observation could be carried out by using cigarettes of varying tar contents, made up to standard specifications, in the TS exposure model used herein. Possible species variation could be studied by the use of SPF rat and hamster and changes in proliferation and differentiation monitored with the spectrum of kinetic techniques as described below.

2. **Development of a spectrum of kinetic techniques for application to material from human airways and those of experimental animals:** a) the thymidine analogue BUdR, incorporated into DNA and labelled by monoclonal antibodies can be visualized with immunoperoxidase staining; combination of this technique with a fluorescein-labelled probe for total DNA would enable

determination of the proportion of cells in each phase of the cycle with a Fluorescence Activated Cell Sorter and the labelling index with flourescence microscopy;

b) use of specific markers eg. Topoisomerase II (Heck and Earnshaw, 1986), to monitor the onset and duration of DNA synthesis; the monoclonal antibody Ki67, to determine the size of the G_0 population (Gerdes et al., 1984);

c) the ^{3}H-T techniques of double labelling and continuous labelling (with careful use), enable determination of the length of the S phase and the size of the growth fraction respectively, for comparison with the values found using other methods.

3. **Application of these techniques to the study of proliferation and differentiation in human airway mucosa:** human airway material from patients with chronic obstructive lung disease, cystic fibrosis as well as suitable control material, is now available and can be maintained as both explants and disaggregated cell preparations in vitro. Information gained from application of the above techniques to these systems, added to quantitative morphological analysis with light and elecron microscopy, will enable the accumulation of valuable data on proliferating populations in these tissues.

4. **Experimental manipulations in vitro:**

a) the role of the secretory cells in proliferation in human tissue could be investigated using the above techniques; a comparison between renewal mechanisms in normal and diseased airways at both proximal and distal levels would be of particular interest.

b) the spectrum of kinetic techniques could be applied to airways from animals made 'bronchitic' by 14 days exposure to TS, which, with appropriate control tissue, were established in vitro.

c) this <u>in vitro</u> system could be used to examine the effects on epithelial proliferation and differentiation in both human and normal and 'bronchitic' rat tissues of:

- growth factors eg. retinoids, TGF-β, cyclic-AMP, EGF and Ca^{++} concentration;
- inflammatory mediators eg. the prostaglandins (PGE_1, PGE_2, PGF_2) and the leukotrienes;
- neuropeptides eg. substance P, VIP, bombesin, somatostatin.

5. **Experimental manipulations <u>in vivo</u>:**

a) in the light of the association between obstructive lung disease and mucous cell hyperplasia in the small airways, it would be of interest to use the spectrum of kinetic techniques to analyse changes in rat peripheral airways due to SO_2 exposure, shown previously to cause mucous cell hyperplasia in this area.

b) it has been demonstrated that non-steroidal and steroidal anti-inflammatory drugs inhibit mucous cell hyperplasia in TS-exposed rats (Rogers and Jeffery, 1986b). Is this effect due to a change in the cell proliferative response or on mucous synthesis and secretion or both? Application of the kinetic techniques described in Section 2. above, to TS-exposed animals given concurrent Flurbiprofen, Indomethacin or suitable steroids eg. Prednisolone or Hydrocortisone could begin to answer these questions.

c) Recently it has been shown that Vitamin E, an anti-oxidant, prevented dimethyl-benzanthracene (DMBA)-induced oral carcinogeneses

in hamster (Trickler and Shklar, 1987). It would be of interest to investigate the effects of Vitamin E on changes in mucous cell number, proliferation and differentiation resulting from TS exposure.

d) in another recent report (Temple and Basu, 1987), β-carotene was shown to have a protective effect against dimethylhydrazine (DMH)-induced carcinogenesis in mouse colon. The epithelial changes due to both excess Vitamin A and TS exposure of Vitamin A-deficient animals, have been described, but the effects of β-carotene or excess Vitamin A on TS-induced epithelial changes in morphology and kinetics have not been studied.

e) The findings regarding the effects of Nac on TS-induced proliferation, differentiation and mucous cell numbers suggest a complex and interesting response that deserves further investigation. It would be informative to determine:

- the glutathione and superoxide dismutase levels in airway epithelial cells exposed to TS and SO_2;
- the changes in these parameters induced by Nac;
- the correlation, if any, with changes in epithelial morphology, proliferation and differentiation.

6. **Mathematical modelling of cell renewal:** preliminary collaborative work has been done to develop an interesting method of mathematical modelling and statistical analysis which will enable data generated from these experiments to be manipulated and analysed in a flexible and informative way. A software package called GLIM (Baker and Neldor, 1978) can be used to construct a heirarchy of models for the data, expressed as progressively more complex equations which can include

factors related to each variable, show the possible relationships of variables to each other and include all potential sources of error. Thereafter the model of best fit is found with the GLIM method of Maximum Liklihood, which uses liklihood ratio tests. The model which best fits the data is the simplest equation which produces the lowest variance; it will indicate the variables with which significant change are associated. GLIM allows this to be done by generating an 'expected' set of values for each equation, then comparing the 'expected' with 'observed' values using analysis of variance. The goodness-of-fit is also compared by a plot of the residual values (ie. 'expected'-'observed') against the fitted values (ie. 'expected'). If the fit is good, this plot is random; if the model does not fit, the plot shows a definite pattern. This method will allow significant differences between control and treated groups to be determined and will also generate equations expressing the behaviour of a particular parameter eg. the number of mucous cells or the growth fraction, with time.

7.7. GENERAL SUMMARY

1. An experimental model of sub-acute tobacco smoke (TS) exposure of SPF rat airway epithelium was used to study mucous cell hyperplasia and (i) examine the contribution of proliferation (Experiments 1 and 2) and differentiation (Experiments 3 and 4) to the response in a number of cell types and (ii) determine the modifying effect on this response, of the drug N-acetylcysteine (Experiment 5).

2. Control pooled data showed that in main bronchial epithelium of healthy control, male, Sprague-Dawley, SPF rats: **ciliated** cells comprised 43%, **serous** cells 24%, **basal** cells 22%, **indeterminate** cells 8%, **transitional** cells 3% and **mucous** cells 0.3% of the cell population.

3. In 3 experiments (1, 2 and 5), proliferation was studied by pulse labelling dividing cells with ^{3}H-T: animals were killed 1h after an intraperitoneal administration of ^{3}H-T. The proliferating population in control rat airway epitheliuum comprised: **basal** cells 71%, **serous** cells 17%, **indeterminate** cells 9%, **transitional** 1% and **ciliated** cells 2%.

4. The data suggested that there is circadian variation in epithelial proliferation, indicated by the greater number of dividing serous cells in the morning compared to the evening.

5. In 3 experiments (3, 4 and 5), differentiation and 'metaplasitic' change were studied by giving a single pulse label of ^{3}H-T on day 0 of the experiment and quantitatively analysing the redistribution of label throughout epithelial cell compartments with time. In control animals, the proportion of the labelled population that was basal decreased while that which was ciliated and transitional (Experiment 4) or mucous (Experiment 3) increased steadily with time; changes in labelled indeterminate and serous proportions mirrored each other. These observations suggest that basal cells give rise to indeterminate and ciliated cells by

differentiation and 'metaplastic' change of serous cells produces indeterminate, transitional and mucus cells.

6. In all experiments, inhalation of TS induced an decrease in the proportion of serous cells, an increase in the mucous cell proportion and a shift from densely stained to weakly stained intracellular granules. The mucous cell increase and shift in granule type were considerably greater in Experiments 1&3 than in Experiments 2&4.

7. These differences were maintained with respect to the proliferating populations: in Experiment 1, TS exposure induced a proliferative response in the indeterminate, transitional and mucous cell compartments, whereas in Experiment 2, a much smaller proliferative response was observed and it occurred in the indeterminate and transitional compartments only.

8. In both differentiation experiments, it was observed that TS exposure accelerated the production of ciliated cells from basal cells so that more ciliated cells were produced at an earlier time. Although TS did not appear to increase the size of the secretory compartment (ie. serous, transitional and mucous cells) due to differentiation, metaplastic change from serous to transitional and mucous cells was observed, in Experiment 1 but not in Experiment 2.

9. TS-induced changes in epithelial proliferation and differentiation and their modification by N-acetylcysteine (Nac), were studied in Experiment 5. TS produced a considerable increase epithelial

labelling comprising a large short-lived increase in basal cell proliferation, followed by a much smaller, sustained increase in mucous cell proliferation.

10. Nac delayed, decreased and attenuated the proliferative response to TS: its major effect was exerted on the basal cell compartment in which the decrease mirrored that of the total labelled population.

11. These findings illustrate and emphasise the importance of the mucous cell as well as the basal cell in the airway response to injury and suggest that cell types active in the proliferative response can vary depending on the nature of the injury.

12. It is suggested that Nac may have its effect by either preventing the decrease in protective intra-cellular enzymes such as the glutathione system or enabling increased synthesis of these enzymes.

APPENDICES

APPENDIX 1.

TABLE A.1.1

EXPERIMENT 5 : PROLIFERATION

- Labelled Epithelial Cell Numbers/10 HPF (± SEM)

NAC-Only

	TIME (days)					
CELL TYPE	0	1	3	7	10	14
CILIATED	0	0.08 (± 0.05)	0.02 (± 0.02)	0	--	0.14 (± 0.05)
SEROUS	0.17 (± 0.06)	0.19 (± 0.07)	0.10 (± 0.04)	0.07 (± 0.04)	--	0
MUCOUS	0	0	0	0	--	0.07 (± 0.03)
TRANSITIONAL						
sero-mucous	0	0	0	0	--	0
muco-serous	0	0	0	0	--	0
INDETERMINATE	0.13 (± 0.08)	0.08 (± 0.05)	0.06 (± 0.04)	0	--	0.03 (± 0.02)
BASAL	0.37 (± 0.09)	0.33 (± 0.1)	0.56 (± 0.2)	0.32 (± 0.1)	--	0.41 (± 0.07)
TOTAL	0.67 (± 0.1)	0.69 (± 0.1)	0.75 (± 0.2)	0.39 (± 0.1)	--	0.66 (± 0.1)

-- = No samples taken.

Statistically significant changes are shown in Figs. 5.2 - 5.8.

TABLE A.1.2

EXPERIMENT 5 : PROLIFERATION

- Labelled Epithelial Cell Numbers/10 HPF (± SEM)

TS-Only

	TIME (days)					
CELL TYPE	0	1	3	7	10	14
CILIATED	0	0.14 (± 0.06)	0.09 (± 0.05)	0.05 (± 0.03)	0.03 (± 0.02)	0.05 (± 0.05)
SEROUS	0.17 (± 0.06)	0.49 (± 0.1)	0.40 (± 0.1)	0.19 (± 0.05)	0.05 (± 0.03)	0.02 (± 0.02)
MUCOUS	0	0.03 (± 0.03	0	0.05 (± 0.03)	0.2 (± 0.07)	0.16 (± 0.08)
TRANSITIONAL sero-mucous	0	0	0	0.02 (± 0.02)	0	0
muco-serous	0	0	0	0.03 (± 0.02)	0.03 (± 0.02)	0
INDETERMINATE	0.13 (± 0.08)	0.16 (± 0.07)	0.05 (± 0.04)	0	0.03 (± 0.03)	0
BASAL	0.37 (± 0.09)	9.06 (± 0.5)	0.35 (± 0.1)	0.57 (± 0.1)	0.55 (± 0.1)	0.17 (± 0.08)
TOTAL	0.67 (± 0.15)	9.85 (± 0.5)	0.84 (± 0.2)	0.88 (± 0.1)	0.9 (± 0.15)	0.4 (± 0.1)

Statistically significant changes are shown in Figs. 5.2 - 5.8.

TABLE A.1.3

EXPERIMENT 5 : PROLIFERATION

- Labelled Epithelial Cell Numbers/10 HPF (± SEM)

NAC + TS

	TIME (days)					
CELL TYPE	0	1	3	7	10	14
CILIATED	0	0.9 (± 0.04)	0.12 (± 0.06)	0.04 (± 0.04)	0.02 (± 0.02)	0.1 (± 0.05)
SEROUS	0.17 (± 0.06)	0.07 (± 0.03)	0.83 (± 0.16)	0.25 (± 0.07)	0.17 (± 0.05)	0.02 (± 0.01)
MUCOUS	0	0.02 (± 0.02)	0.17 (± 0.06)	0.07 (± 0.04)	0.12 (± 0.04)	0.35 (± 0.08)
TRANSITIONAL sero-mucous	0	0	0	0.08 (± 0.05)	0.05 (± 0.04)	0
muco-serous	0	0	0.09 (± 0.04)	0.1 (± 0.06)	0.02 (± 0.02)	0.02 (± 0.02)
INDETERMINATE	0.13 (± 0.08)	0	0.2 (± 0.08)	0.09 (± 0.04)	0.	0
BASAL	0.37 (± 0.09)	0.18 (± 0.06)	1.9 (± 0.3)	1.7 (± 0.25)	0.39 (± 0.08)	0.4 (± 0.1)
TOTAL	0.67 (± 0.15)	0.32 (± 0.08)	3.3 (± 0.48)	2.4 (± 0.27)	0.7 (± 0.1)	0.95 (± 0.1)

Statistically significant changes are shown in Figs. 5.2 - 5.8.

APPENDIX 2.

PROCESSING TISSUE FOR ELECTRON MICROSCOPY

1. primary fixation: 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) - 2 hours.

2. rinse: 4 X 10 minutes 0.1M sodium cacodylate buffer (pH 7.4)

3. secondary fixation: 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) - 2 hours

4. rinse: 4 X 10 minutes - 0.1 M sodium cacodylate buffer (pH 7.4)

5. dehydration: 3 X 1 min 50% methanol
   3 X 3 min 70% methanol
   3 X 3 min 95% methanol
   4 X 15 min 100% methanol
   2 X 15 min epoxy propane

6. infiltration: 2 X 30 min 50/50 epoxy propane/araldite
   overnight: 100% araldite
   4 h fresh 100% araldite

7. embedding: fresh 100% araldite @ 60°C for 2 days.

APPENDIX 3.

REAGENTS USED FOR TISSUE PROCESSING

25% Glutaraldehyde purified for electron microscopy

Sodium cacodylate buffer

Osmium tetroxide crystals: 1 g ampoules

Spurr's embedding mixture: Vinyl cylcohexene dioxide (resin)
Nonenyl succinic anhydride (hardener)
DER 736 (plasticiser)
Dimethylamino ethanol (accelerator)

Araldite 502: 502 resin
DDSA
DMP-30

All products were obtained from EM-Scope Laboratories Ltd.,
London, UK.

APPENDIX 4.

ADDRESSES OF SUPPLIERS (ALPHABETICAL LISTING)

Amersham International Pty. Ltd., Amersham Place, Little Chalfont, Buckinghamshire, HP7 9 NA.

Analysis Automation Ltd., Southfield House, Eynsham, Oxan, OX8 IJD.

BDH (British Drug Houses) Chemicals Ltd., Freshwater Road, Dageham, Essex, RM8 IRZ.

Charles Austen Pumps Ltd., 100 Royston Road, Byfleet, Weybridge, Surrey, KT14 7PB.

Charles River U.K. Ltd., Manston Road, Margate, Kent, CT9 4LT.

EM Scope, London, 374 Wandsworth Road, London, SW8 4TE, U.K.

Energetics Science Inc.: U.K. distributors - Anaysis Automation Ltd.

Forth Tec Services Ltd., Mayfield, Dalkeith, Midlothian, Scotland, EH22 4AQ.

Gallenkamp and Co. Ltd., P.O. Box 290, Technico House, Christopher Street, London, EC2 2ER.

Heygate and Sons Ltd., Bugbrooke Mills, Northampton, Northants., NN7 3QH.

Ilford U.K. Ltd., Town Lane, Mobberley, Knatsford, Cheshire, WA16 7HA.

Instrumentation Laboratory: U.K. distributors - Analysis Automation Ltd.

Kodak Ltd. U.K., P.O. Box 66, Hemel-Hempstead, Herts., HP1.

E. Leitz (Instruments) Ltd., 48 Park Street, Luton, LU1 3HP.

L.K.B. - Biochrom, Cambridge Science Park, Milton Road, Cambridge.

May and Baker Ltd., Rainham Road South, Dagenham, Essex, RM10 7XS.

Olac, Shaws Farm, Bicester, Oxon., OX6 OTP.

Raymond A. Lamb, 6 Sunbeam Road, London, NW10 6JL.

Zambon S.p.A. (Milan), Corporate Medical Dept., via L. Del Duca, 10, 20091 Bresso (MI), Italy.

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