Enhanced Growth Potential of Cultured Rabbit Tracheal Epithelial Cells Following Exposure to N-Methyl-N'-nitro-N-nitrosoguanidine

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To establish a standardized model for the transformation of rabbit airway epithelial cells, we attempted to transform rabbit tracheal epithelial (RbTE) cells in culture with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). RbTE cells, harvested by enzymatic digestion from male New Zealand white rabbits, were plated onto feeder layers of irradiated 3T3 cells. Control cells proliferated exponentially during the 2nd week of culture and reached the plateau phase by the 3rd week. Cells exposed to MNNG (0.1 μg/ml) proliferated in a fashion similar to the control cells, except that there was some delay before proliferation began. The clonogenic activity of RbTE cells rapidly decreased in parallel with the increase in cell population equally in the control and MNNG groups. During the late plateau phase, cells exposed to MNNG regained clonogenic activity, and this compartment size expanded with time, whereas the clonogenic activity in control cultures remained below the detectable level. In RbTE cell cultures exposed three times to 0.1 μg/ml MNNG, large, persistent and proliferating colonies emerged at a frequency of $1-3 \times 10^{-2}$ among the surviving clones, whereas all the control cultures eventually became senescent. The MNNG-induced alteration in the growth potential of RbTE cells, i.e., the extended lifespan, and the maintenance and even expansion of clonogenic activity, was similar to that of transformed rat tracheal epithelial cells. However, no immortal cell line could be established from these growth-altered RbTE cells. We therefore concluded that the growth-altered RbTE cells were partially transformed.

Key words: Tracheal epithelial cell — Rabbit — Culture — Chemical carcinogen — Enhanced growth potential

The airway epithelium is composed of diverse cell types with different functions and cell kinetics. In the study of pulmonary carcinogenesis, it is essential to clarify the role of specific cell types in terms of carcinogen metabolism, as well as in terms of sites of carcinogenic insult. The rabbit is an animal suitable for isolation of specific types of epithelial cell from the respiratory tract, and studies with cells isolated from rabbits have proven to be useful in elucidating the roles of such cell types in pulmonary carcinogenesis.[1-6] For example, the metabolization of xenobiotics and chemical carcinogens has been investigated in Clara cells and type II pneumocytes isolated from rabbit lungs.[1-3] The growth and differentiation potential of tracheal basal and Clara cells isolated from rabbits have also been investigated.[4-6] Thus, an experimental approach to transform *in vitro* such specific types of epithelial cell isolated from the rabbit airway should yield interesting results.

Rabbit tracheal epithelial (RbTE) cells can be readily cultured and have been extensively used to study the cellular, biochemical, and molecular mechanisms of mucous and squamous differentiation.[7,8] They can be easily manipulated to differentiate as either mucociliary or squamous epithelium, depending on the presence or absence of retinoids in the medium.[8] They might, therefore, serve as a particularly interesting tool for examination of the relationship between differentiated phenotype and transformability. Studies of *in vivo* tracheal injection of chemical carcinogens in rabbits have resulted in the induction of both squamous cell carcinomas and adenocarcinomas.[9,10]

We carried out this study to determine whether RbTE cells could be transformed in culture in a manner similar to rat tracheal epithelial (RTE) cells, which have been extensively used to study multistage neoplastic transformation.[11] We believe that the results of these experiments could help to establish a standardized *in vitro* transformation model that could be employed for various types of airway epithelial cells isolated from rabbits. In this context, we performed cytotoxicity studies of RbTE cells exposed to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Subsequently, we attempted to transform the primary RbTE cells in culture with appropriate MNNG treatment. In these experiments, we evaluated the

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MNNG-induced changes in proliferative activity of RbTE cells by assessing the cell population, clonogenic activity and subculturability. We used MNNG in this study because this chemical is a direct-acting carcinogen that does not require metabolic conversion to be activated, and because we can compare our results with the data in RTE cell transformation experiments, in which MNNG has been extensively used.[11]

MATERIALS AND METHODS

Preparation of primary RbTE cells The method used for the preparation of RbTE cells has been described elsewhere.[12] Briefly, tracheas were removed from 2- to 3-month-old male New Zealand white rabbits (Dutchland Laboratory Animals, Denver, PA), and the tracheal lumen was filled with 0.1% protease (Sigma, St. Louis, MO, type XIV) in Ham's F12 medium (Gibco Laboratories, Grand Island, NY). The tracheas were incubated at 4°C overnight and the lumens were flushed with Ham's F12 containing 5% fetal bovine serum (FBS, Gibco). The cell suspension was then filtered through nylon mesh (Tetko Inc., Elmsford, NY, HC-100) and centrifuged at 150g for 10 min at 4°C. Cell pellets were resuspended in culture medium containing 5% FBS.

Cell culture and carcinogen treatment The method used for culturing RbTE cells was essentially the same as that used for RTE cells.[13] Briefly, Swiss 3T3 cells (J-2 strain), kindly provided by Dr. Howard Green, Massachusetts Institute of Technology, Boston, MA, were lethally irradiated at 50 Gy using a ^{137}Cs source (J.S. Shepard Assoc., Glendale, CA) and were plated at a density of 4×10^5 cells per 60-mm dish (Falcon, Oxnard, CA) in Ham's F12 medium. The medium contained the following antibiotics and supplements at the final concentrations indicated; penicillin (Gibco, 60 IU/ml), streptomycin (Gibco, 60 μg/ml), human transferrin (Sigma, 10 μg/ml), bovine insulin (Sigma, 10 μg/ml), hydrocortisone (Sigma, 0.1 μg/ml), epidermal growth factor (Collaborative Research, Waltham, MA, 25 ng/ml) and 5% FBS. Twenty-four hours later, primary RbTE cells were plated onto the feeder layers at clonal densities (0.3–5 $\times 10^4$ cells per dish), and allowed to attach overnight. The following day, cultures were exposed to MNNG or to control vehicle alone. MNNG was obtained from the Chemical Repository, Chemical Carcinogenesis Program, NCI, Bethesda, MD, and stock MNNG solution in absolute ethanol was kept at -20°C until use.[13] Just prior to use, the stock MNNG solution was diluted in Ham's F12 buffered with 20 mM N-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulfonic acid] (HEPES, Gibco) (pH 6.8). The culture medium was aspirated 24 h after the RbTE cells were plated, and the MNNG solution was applied to the cultures for 4 h. In some experiments,

MNNG was applied three times, for 4 h each time, at 24, 48 and 72 h. After the cultures had been exposed to MNNG, the medium was replaced by fresh medium. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed twice a week.

Assessment of MNNG cytotoxicity The cytotoxic effects of MNNG on RbTE cells were assessed in the same manner as for RTE cells.[13] Seven days after the cultures were exposed to MNNG, the cells were fixed in absolute methanol and stained with a 10% Giemsa solution. The dishes were observed under a dissecting microscope, and epithelial cell colonies consisting of 50 or more cells were counted to determine colony-forming efficiency (CFE). Relative survival was calculated as the proportion of the CFE with specified concentrations of MNNG against the CFE of the control cultures.

Assessment of cell number and colony-forming activity in cultures After the primary RbTE cells (5×10^3 cells per 60 mm-dish) were exposed to 0.1 μg/ml MNNG, the number of cells in the cultures, as well as the frequency of cells capable of forming colonies on feeder layers, were determined at 7, 10, 13, 17, 21, 25, and 29 days. Before 7 days, the epithelial cell colonies were too small to determine exactly the number of cells present in the cultures. After the medium was aspirated, the cultures were washed with Ca^{2+}-,Mg^{2+}-free phosphate-buffered saline (PBS, Gibco) (pH 7.3), then feeder cells were removed as completely as possible by brief incubation and flushing of cultures with 0.002% ethylenediaminetetraacetic acid (EDTA, Gibco) in Ca^{2+}-,Mg^{2+}-free PBS (pH 7.3).[13] The epithelial cells which then remained were harvested by treatment with 0.05% trypsin (Gibco) and 0.002% EDTA in Ca^{2+}-,Mg^{2+}-free PBS (pH 7.3) and were counted with a hemocytometer. Viability was determined by the Trypan Blue dye exclusion test. The harvested RbTE cells were replated onto feeder layers to determine the frequency of colony-forming cells. Seven days later, the cultures were fixed and stained for counting the number of colonies.

Transformation experiment The primary RbTE cells on feeder layers (5×10^4 cells per 60 mm-dish) were exposed three times to 0.1 μg/ml MNNG, then the medium was replaced with fresh medium and the cultures were maintained for up to 6 weeks. The morphology of cells in culture was observed periodically with a phase microscope. When required, cultures were fixed and stained with Giemsa for light microscopic examination. Sets of dishes were employed for the determination of cell number and for determining the frequency of colony-forming cells on feeder layers. Subculturability was tested by replating cells onto either feeder layers or a plastic substratum at high cell densities (0.5–1 $\times 10^6$ cells per 60-mm dish) to detect cells whose growth characteristics had changed.

RESULTS

Cytotoxic effects of MNNG on RbTE cells The cytotoxicity of MNNG towards RbTE cells was determined by calculating the frequency of colony-forming cells that survived treatment with various concentrations of MNNG relative to that of the control-treated cells. The frequency of colony-forming cells among the control primary RbTE cells ranged from 1.0% to 2.0%. As shown in Fig. 1A and 1B, there was a typical dose-dependent reduction in the frequency of surviving colony-forming cells, and therefore in the relative survival as well, with increased MNNG doses. The results of several experiments showed that a single exposure of RbTE cells to 0.1 µg/ml MNNG resulted in 25% to 50% relative survival (Fig. 1B).

The cytotoxic effects of multiple exposure to MNNG were also studied by exposing RbTE cells to various concentrations of MNNG at 1, 2, and 3 days; at each time 1/3 of the MNNG dose in a corresponding single exposure was applied. Such triple exposure was less toxic than a single exposure to the cumulative dose (Fig. 1B). Triple exposure of RbTE cells to 0.1 µg/ml MNNG resulted in 1.3% to 7.0% relative survival.

Morphology of RbTE cells in culture In the control cultures, small epithelial colonies appeared at 4 to 5 days after seeding, the colonies rapidly increasing in size due to florid cell replication. Almost all the cells in such proliferating colonies were small and round, and had high nuclear:cytoplasmic ratios. Cell stratification was observed in some large colony areas after 10 days. By 14 days after the beginning of culture, colony expansion had almost ceased, and numerous flattened large cells had appeared within the colonies. Such large flattened cells increased in number with further time in culture.

When the cultures were exposed to 0.1 µg/ml MNNG, colonies were visible at 6 to 7 days, 2 to 3 days after colonies were first seen in controls. These surviving colonies, which were considerably smaller than those in the control cultures, began to grow rapidly in the 2nd week. By 14 days, their morphology, in terms of colony size and cell composition, was indistinguishable from that of the control colonies.

Cell growth curve RbTE cells (5×10^3 cells per dish) were treated with MNNG (0.1 µg/ml) or vehicle alone, and the number of cells present in the cultures was determined at various time points from 7 to 29 days. As shown in Fig. 2, in controls there was a rapid growth
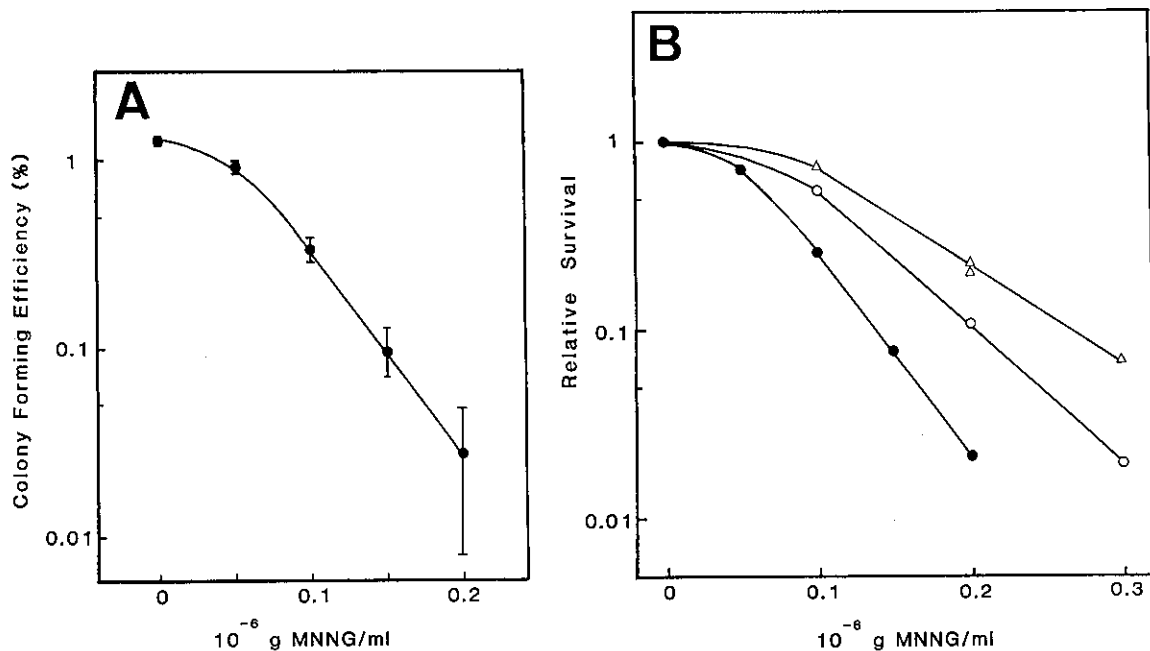

Fig. 1. (A) Representative cytotoxic dose response of RbTE cells to a single exposure of MNNG, showing typical dose-dependent reduction in colony-forming efficiency, with a shoulder curve in the lower range of the MNNG dose. Values represent the mean±SD of 5 dishes at each dose. The colony-forming efficiency of control cells ranged from 1% to 2% in several experiments. (B) Cytotoxic dose response of RbTE cells to MNNG; ●, single exposure at one day; ○, single exposure at 3 days; and △, triple exposure at 1, 2, and 3 days. Triple exposure doses of MNNG are regarded as cumulative doses. Note that single exposure to MNNG at one day is most toxic, and split triple exposure the least toxic. Values represent the mean of 5 dishes at each dose, and the SD is within the range of ±5% of the mean value.
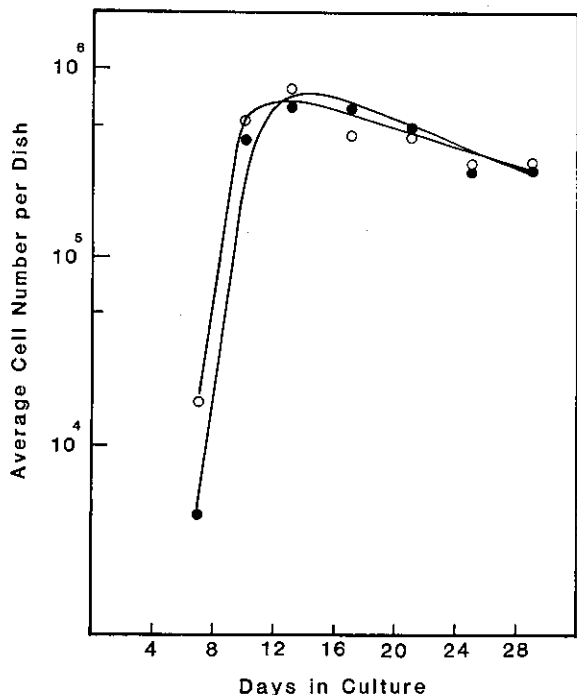
Fig. 2. Growth curve of RbTE cells after treatment with 0.1 μg/ml MNNG (●) or with vehicle alone (○). Note that the exponential growth phase occurs between 7 and 13 days in the control and MNNG groups, with some delay in cultures exposed to MNNG. Cell numbers during the plateau phase are essentially the same in both groups. Values represent the mean of 5 replicate dishes at each time point, and the SD is within the range of ±5% of the mean value.
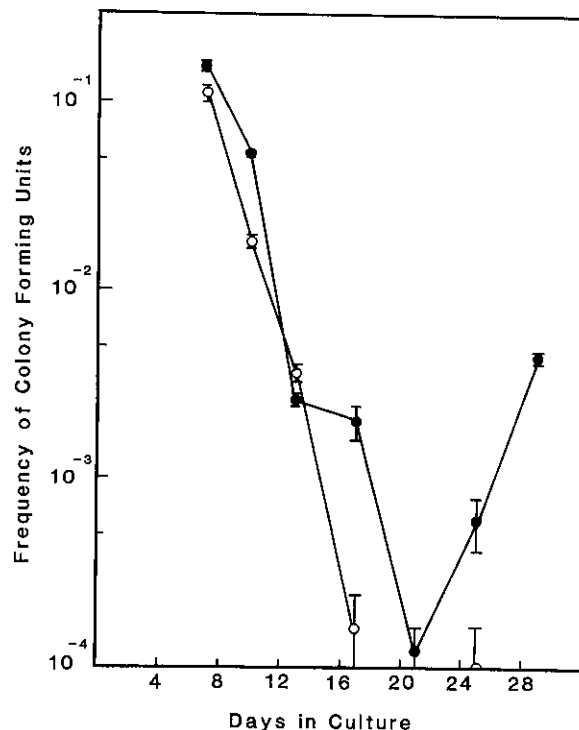

Fig. 3. Changes in frequency of colony-forming units (cells) with time in culture after treatment with 0.1 μg/ml MNNG (●) or with vehicle alone (○). The frequency of colony-forming units in cultures shows an exponential decrease with time between 7 and 17 days in the control and MNNG groups. The frequency of colony-forming units increased from 10^{-4} at 21 days to 5×10^{-3} at 28 days in the cultures exposed to MNNG, whereas it remained below 10^{-4} in the control cultures. Values represent the mean±SD of 5 replicate dishes at each time point.

phase between 7 and 10 days, following which the number slowly began to decline (13 to 29 days). The cultures exposed to MNNG followed a very similar pattern, except that they started at a lower level due to the MNNG toxicity.

Colony-forming activity RbTE cells (5×10^3 cells per dish) were treated with MNNG (0.1 μg/ml) or vehicle alone, and the frequency of colony-forming cells present in the cultures was determined as a function of time at various time points from 7 to 29 days. As shown in Fig. 3, the frequency of colony-forming cells (CFE) in the control cultures decreased exponentially, from 10^{-1} at 7 days to 10^{-4} at 17 days, and it then remained at around or below that level for the duration of the experiment. In cultures exposed to MNNG, the CFE decreased in the same fashion and to the same level as in the control for up to 14 days. However, by 17 days the CFE was consistently 10-fold larger than that of the control. The CFE in the MNNG-treated cultures continued to decrease up to 21 days, at which time it

increased dramatically, reaching approximately 500-fold the control value at 29 days. These results indicated that colony-forming activity was restored in the MNNG-treated cultures, and that it even increased with time.

Subculturability To detect cells whose growth had been altered, we carried out extensive subculture studies. At various time intervals, cells from cultures exposed to 0.1 μg/ml MNNG were pooled and replated, either onto feeder layers or onto a plastic substratum, at high densities. On occasion, cultures exposed to MNNG were subcultured 2 or 3 times, while controls could not be subcultured.

Persistent colonies after multiple exposure to MNNG RbTE cell transformation was attempted by multiple exposure of 5×10^4 cells per dish to 0.1 μg/ml MNNG at 1, 2, and 3 days after plating. This dose of MNNG was markedly toxic and killed most cells (relative survival 1.3% to 7.0%). Around 5 weeks after exposure to

MNNG, there emerged in each dish one to three huge colonies (Fig. 4) composed of both small and large flattened cells (Fig. 5A). In each dish of the controls, there were ten to thirty small colonies (Fig. 4) composed exclusively of large flattened cells (Fig. 5B). Huge colonies were still growing in the cultures that had been exposed to MNNG at this time, whereas colonies in the control cultures showed no evidence of enlargement. Cells in the control cultures desquamated and the cell density in the colonies became lower with time. For the determination of frequency of colony-forming cells in the cultures, RbTE cells were dissociated and replated onto feeder layers at 6 weeks. None of the eleven control cultures contained colony-forming cells at a detectable level ($< 10^{-5}$), whereas three of the seven cultures exposed to MNNG contained colony-forming cells, at a frequency of 3×10^{-4} to 1.3×10^{-2}. There was no significant difference in cell number between the control and MNNG groups (4.9–6.2×10^5 cells per dish). Subculturability was examined by plating pooled cells, but the cells in the control and MNNG groups eventually senesced.
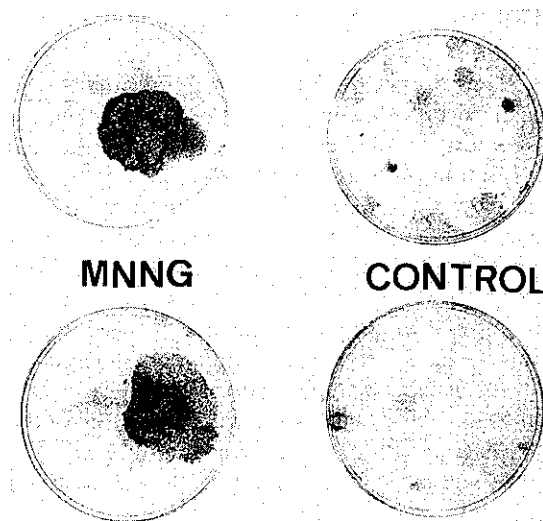

Fig. 4. Giemsa-stained RbTE cell cultures at 6 weeks after triple exposure to 0.1 μg/ml MNNG or vehicle alone. The culture exposed to MNNG (MNNG) contains a few large dark-stained colonies, while the control culture (Control) contains many small, lightly-stained colonies.
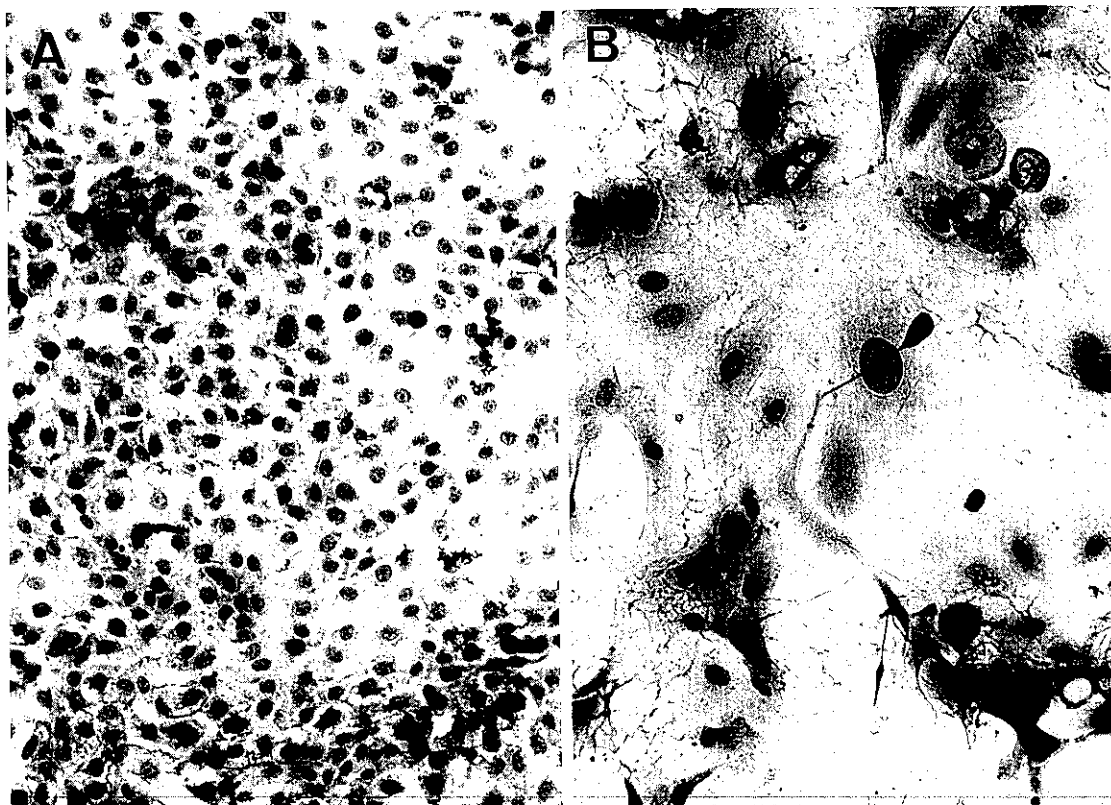

Fig. 5. Light microscopy of RbTE cell colonies at 6 weeks after triple exposure to 0.1 μg/ml MNNG (A) or vehicle alone (B). Colonies in the culture exposed to MNNG consist of tightly packed small round cells with a high nuclear:cytoplasmic ratio (A), while colonies in the control cultures consist exclusively of large flattened cells (B). Giemsa stain, $\times 75$.

DISCUSSION

The primary purpose of this study was to establish a standardized culture method for the transformation of RbTE cells, a method which would be applicable for the transformation of various types of airway epithelial cells isolated from the rabbit.

The cytotoxic effect of MNNG was determined as the fraction of cells that survived the MNNG-treatment and formed colonies. We believe that the most reliable index of cell survival is colony formation, because only cells that survive and proliferate can be considered in the quantification of carcinogen-induced changes.[13] The susceptibility of RbTE cells to the MNNG cytotoxicity was almost equal to that of RTE cells.[13]

MNNG induced significant alterations in the proliferative activity of RbTE cells. This included the regaining of self-renewal capacity followed by an expansion of the clonogenic cell population, increased subculturability of the cells, and the appearance of persistent huge proliferative colonies containing significant numbers of clonogenic cells. The biological properties of such growth-altered RbTE cells are in many respects similar to those of transformed RTE cells in the earliest stage.[14-17]

Extensive transformation studies with RTE cells have revealed that at least three steps are required for the primary RTE cells to become neoplastic: (1) the appearance of large proliferating colonies, (2) the establishment of immortal cell lines, and (3) the appearance of neoplastic variants.[11] Transformed colonies of RTE cells are termed enhanced growth variants and are the earliest phenotype recognized in RTE cell transformation.[11] The cells comprising such enhanced growth variant colonies are biologically characterized by continuous clonal expansion, and by maintenance, or even expansion, of the clonogenic cell compartment.[15-17] Another important biological property of transformed RTE cells is the frequent production of immortal cells following several subcultures; the appearance of these cells is associated with the appearance and. expansion of variant clonogenic cells that are capable of growing on a plastic substratum.[16, 17]

The present study showed that exposure of RbTE cells to MNNG induced enhanced growth variants similar to those of RTE cells. However, there was no evidence that the growth-altered RbTE cells had the potential to gain immortality; why this is so is unclear. In the RTE cell system, it has been shown that the variant cells that are capable of growing on plastic and are essential for immortalization emerge spontaneously at a relatively high frequency from the cell population of enhanced growth variant colonies.[16, 17] It is conceivable that the frequency of spontaneous conversion of growth-altered RbTE cells to variant cells capable of replicating in an unlimited fashion on a plastic substratum is quite low; thus, such variant cells could not be detected in the present study. The genetic mechanism involved in transformation and immortalization may show species differences. One feature of human cell transformation, for example, is the extremely low frequency of the acquisition of immortality compared to rodent cells,[18] and this may be associated with the relatively greater karyotypic stability of human cells.[19]

As shown in the present study, the MNNG-induced enhancement of the growth potential of RbTE cells was not sufficient to produce immortal cells. To obtain fully transformed RbTE cells, we should conduct experiments in which different chemical carcinogens are used as transforming agents, and/or superimposed mutagenic agents or inhibitors of DNA repair are applied to the growth-altered cells.

The results of the present study suggest that *in vitro* transformation experiments using Clara cells, type II pneumocytes, or basal cells isolated from rabbits would be extremely useful to investigate which cell type(s) is susceptible to the cytotoxicity and transforming activity of chemical carcinogens, and what phenotypic changes are induced in these cells by the carcinogen treatment. Repopulation of denuded rat tracheas would also be useful for further investigation of the biological properties of such growth-altered cells, since this system provides optimal conditions for isolated or cultured cells to grow and differentiate.[4-6]

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