Repeated allergen inhalations induce DNA synthesis in airway smooth muscle and epithelial cells in vivo

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Panettieri, Reynolds A., Jr., Richard K. Murray, Andrew J. Eszterhas, Gulsevil Bilgen, and James G. Martin. Repeated allergen inhalations induce DNA synthesis in airway smooth muscle and epithelial cells in vivo. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L417–L424, 1998.—Airway smooth muscle (ASM) mass appears to be increased in the bronchi of patients with chronic severe asthma. Although the precise mechanisms that induce these changes are unknown, increases in ASM mass are caused, in part, by ASM cell proliferation. After allergen challenge in rats, it has been possible to demonstrate an increase in ASM mass by morphometric techniques. To examine whether hyperplasia is involved in ASM cell growth in vivo, we investigated whether repeated allergen challenges in sensitized Brown Norway rats stimulated DNA synthesis in airway epithelial and ASM cells. Animals that were actively sensitized to ovalbumin (OA) received either three aerosolized OA or saline challenges at 5-day intervals. DNA synthesis was measured by indirect immunohistochemical techniques with an anti-bromodeoxyuridine (BrdU) antibody. OA inhalations increased ASM mass as determined by morphometry and also induced DNA synthesis in both airway epithelial and ASM cells in the airways of sensitized animals compared with saline-challenged control animals. ASM mass was increased in large- and medium-sized airways but not in small airways. However, the number of BrdU-positive ASM cells normalized to basement membrane length was also greater in the large- and medium-sized airways compared with that in the small airways. When the number of BrdU-positive epithelial cells was normalized to basement membrane length, there was no difference among airway sizes and the number of BrdU-positive epithelial cells. These data suggest that DNA synthesis is induced in both airway epithelial and ASM cells after inhalational antigen challenge.

AN INCREASE IN THE QUANTITY OF AIRWAY SMOOTH MUSCLE (ASM), which is due to an increase in airway myocyte number (hyperplasia) as well as to ASM cell hypertrophy, has been described in the airways of patients with chronic severe asthma (6, 7, 11, 13, 14). These chronic changes in ASM may be induced by stimulation of airway myocytes with contractile agonists, inflammatory mediators, and growth factors (see Ref. 19 for a review). Airway remodeling of this kind may contribute, in part, to the relative refractoriness of certain asthmatic subjects to medications such as bronchodilators and anti-inflammatory agents that are used to treat airflow obstruction (3, 19, 20). The relationship between airway wall thickening or increases in ASM mass and hyperresponsiveness remains uncertain because both of these changes may conceivably be consequences of long-standing asthma and are not necessarily the cause of hyperresponsiveness. However, if the contractile properties of the increased mass of smooth muscle in asthmatic airways are similar to those in normal muscle, it seems likely that the increased force-generating capacity of the muscle may account for the exaggerated airway narrowing characteristic of this condition.

Although recent in vitro studies have focused on understanding cellular mechanisms that regulate ASM cell proliferation, little information is available with respect to factors that promote ASM cell proliferation or that modulate myocyte growth in vivo. These mechanisms may be quite different in vitro because ASM in culture becomes an actively replicating tissue, in contrast to its apparently quiescent state in vivo (12). To examine these mechanisms, an in vivo model of ASM cell hyperplasia would be useful. Ideally, such a model should be characterized by airway hyperresponsiveness to inhaled contractile agonists and by an increased ASM mass that is a consequence of ASM cell hyperplasia. Previous studies (23, 24) have shown that Brown Norway (BN) rats sensitized to ovalbumin (OA) and repeatedly challenged with antigen exhibit airway hyperresponsiveness and increased bronchial smooth muscle mass compared with rats sensitized to OA and repeatedly challenged with saline. However, the morphometric techniques used to quantitate ASM did not differentiate between hypertrophy and hyperplasia. The aim of the present study was to determine whether the observed increase in ASM after allergen challenge is due to ASM hyperplasia. To this end, the incorporation of bromodeoxyuridine (BrdU), a thymidine analog, was used to determine whether DNA synthesis was stimulated in airway cells. We compared the results of the incorporation of BrdU into airway cells with morphometry, which was used to evaluate ASM mass. In addition to providing information concerning the mechanisms of allergen-induced increases in ASM, the incorporation of BrdU has the potential to be an advantageous alternative to the rather cumbersome morphometric approach. The latter is also subject to observer error because of its dependence on adequate staining to distinguish airway tissues.

METHODS

Sensitization and antigen challenge protocol. A group of 12 BN rats, 7–8 wk old and 130–240 g in body weight, was actively sensitized by a single subcutaneous injection of 1 mg...
of OA and 200 mg of aluminum hydroxide. Simultaneously, 1 ml of Bordetella pertussis vaccine containing $6 \times 10^6$ heat-killed organisms was given intraperitoneally as an adjuvant. Subsequently, the animals received either three OA challenges at 5-day intervals (on days 14, 19, and 24; n = 6 rats) or three challenges with aerosolized saline (n = 6 rats) as previously described (2, 23). Aerosol challenges with OA or saline were performed with aerosols generated by a Hudson nebulizer connected to a side port of the box and driven by compressed air to produce an output of 0.18 ml/min. Aerosols of saline or OA were inhaled by spontaneously breathing animals over a 5-min period. OA was diluted in saline (5% solution) immediately before each experiment.

A schematic illustration of the time schedule for sensitization and challenge is shown in Fig. 1. DNA synthesis was determined in animals injected with 25 mg/kg of BrdU. Cells that enter the S phase of the cell cycle incorporate BrdU, and positive nuclear staining of these cells can be detected by immunocytochemical staining techniques (10). As Hershenson et al. (12) and Pilewski et al. (22) previously described, the timing as well as the dose for the BrdU injections was optimized to obtain maximal labeling of airway epithelial and ASM cells (12, 22).

Tissue preparation. The first three rats that received OA challenges and three saline-challenged control animals were selected for detailed immunohistochemical studies. Three additional animals were added to each group for morphometric studies. Twelve hours after the last BrdU injection, the rats were exsanguinated by cardiac puncture, the lungs were removed for histological studies. The lungs were fixed with an intrabronchial perfusion of 10% neutral Formalin at a constant pressure of 25 cmH$_2$O and at room temperature for a period of 48 h. Morphometry and immunocytochemistry were performed on 5-µm-thick paraffin-embedded sections (micromet model 520, American Optical, New York, NY) taken from the midsagittal regions of each lung. The slides were either stained with hematoxylin-phloxine-saffron (HPS) or processed for immunohistochemistry. Where possible, immunocytochemical and morphometric analyses were performed on adjacent sections.

Analysis of DNA synthesis by anti-BrdU staining. The methods for immunocytochemical staining to detect BrdU incorporation in vivo are identical to those previously reported by Hershenson et al. (12) and Pilewski et al. (22). Tissue sections were deparaffinized with xylene and rehydrated with graded alcohol washes. Endogenous peroxidase activity was quenched with 3% H$_2$O$_2$, and the sections were treated with 0.4% pepsin, washed, and then treated with 3.3 N HCl. After rigorous washes, nonspecific staining was blocked with 1% horse serum, and a murine anti-BrdU monoclonal antibody (1:12.5; Becton Dickinson) was added for 1 h at 37°C. Subsequently, the slides were washed and treated with a biotinylated anti-mouse antibody (1:200; Vectastain). Positive nuclei were identified by developing the slides with a Vectastain ABC reagent kit. In some experiments, sections were stained either with 4,6-diamidino-2-phenylindole (1:2,500), a fluorescent nuclear stain that stains all nuclei, or with a murine anti-CD45 antibody (1:200; BRA-11; Sigma) that identifies cells of leukocyte lineage.

Characterization of cells as airway epithelial or ASM cells. ASM cells were characterized as elongated cells with fusiform nuclear profiles that were located below the basement membrane, were within the muscular layer as determined on consecutive sections stained with HPS, and were not stained with the anti-CD45 antibody. In some studies, BrdU incorporation was determined in vascular smooth muscle. Vascular myocytes were characterized as fusiform, elongated cells in small muscular arteries beneath the endothelial layer that did not stain positively with anti-CD45.

Airway epithelial cells were characterized as columnar cells that were located above the basement membrane and within the epithelial layer as determined on consecutive sections stained with HPS and were not stained with the anti-CD45 antibody. Cells in the epithelial layer, which stained positively with anti-CD45, were considered of leukocyte lineage and were excluded from the analysis.

Morphometric analysis of ASM mass. Measurements of ASM mass were performed as previously described (23). Briefly, measurements of the airway were obtained by tracing the microscopic images of interest onto paper with a drawing tube attachment (Leitz). The outlines of the airway structures were subsequently measured with a computer-controlled digitizing board (Jandel Scientific, Corte Madera, CA) and commercial software (SigmaScan, Jandel Scientific). The airways were analyzed consecutively on slides from left midsagittal sections until a representative sample was obtained. When the number of large airways was very small, the right midsagittal section was also analyzed. Airways with a ratio of maximum internal diameter to minimum internal diameter $\geq 2$ were considered to be cut tangentially and were not measured. The internal diameters were measured from the basement membrane on one side of the airway to the basement membrane on the opposite side. All airways were evaluated for the following morphometric dimensions: length of the airway basement membrane of the epithelium (L$_{bm}$) and area of the ASM. In other experiments, L$_{bm}$ was confirmed by direct image analysis with an Image-1 AT system ($\times$250; Universal Imaging, West Chester, PA).

Antibodies and reagents. OA (grade V) was purchased from Sigma Immunochemicals (St. Louis, MO). Bordetella pertussis vaccine was obtained from the Armand Frappier Institute (Laval des Rapides, Quebec, Canada). Anti-BrdU was obtained from Becton Dickinson, and anti-CD45 was obtained from Sigma. All other reagents were obtained from Sigma unless otherwise stated.

Data analysis. Airways were grouped into three categories based on the L$_{bm}$: large ($>2,000 \mu m$), medium (1,000–2,000 $\mu m$), and small ($<1,000 \mu m$). DNA synthesis was reported as the number of BrdU-positive nuclei from airway epithelial or ASM cells according to airway size. All cell-counting analyses were performed by two observers blinded to the group status. The intraobserver and interobserver variabilities were tested, and the correlations between results were statistically significant. Morphometric analyses were performed by a single individual, also blinded to group status. Data are expressed as means ± SE; statistical comparisons were performed with analysis of variance (Bonferroni-Dunn test), and a P value $< 0.05$ was considered significant.
To determine whether BrdU incorporation in airway cells was dependent on airway size, BrdU-positive cells were normalized to the \( L_{bm} \). Because airway epithelial cells in rat bronchi form a uniform monolayer, BrdU-positive epithelial cells were normalized to the \( L_{bm} \) (in \( \mu m \)). The ASM layer, however, is characterized by multiple layers of airway myocytes so that the number of myocytes is likely to vary as a function of the area of the airway rather than simply of its internal perimeter. Thus BrdU-positive ASM cells were normalized to the square of the \( L_{bm} \) (in \( \mu m^2 \)) to correct for differences in airway size.

**RESULTS**

Characterization of airway size between allergen-challenged and control animals. Analysis of DNA synthesis as measured by BrdU incorporation was performed on lung sections from animals that were actively sensitized and received three successive aerosolized OA challenges (\( n = 3 \) rats) compared with those that were sensitized and received saline (\( n = 3 \) rats). Based on the length of the basement membrane, airways were grouped into three categories: large (>2,000 \( \mu m \)), medium (1,000–2,000 \( \mu m \)), and small (<1,000 \( \mu m \)). All airways from OA-challenged and from control animals were then pooled. Analysis was performed on a total of 204 airways (34 airways/category) or 34 airways/animal. There was no significant difference in the size of the airways studied between OA-challenged and control animals (\( P > 0.05 \) by analysis of variance; Fig. 2).

Repeated allergen challenges induced DNA synthesis in airway epithelial cells and ASM cells. Representative light photomicrographs of a medium-sized airway from a control and an OA-challenged animal are shown in Fig. 3. In both the control and OA-challenged animals, the airways typically show a thin ASM cell layer and a one-cell-thick epithelium (Fig. 3A). After repeated allergen challenges, there were marked increases in the number of airway cells that stained positively for BrdU compared with animals challenged...
with saline (Fig. 3, B and D). To confirm that the nuclei in cells from the control animals were intact, tissue sections were also stained with 4,6-diamidino-2-phenylindole, which is a nonspecific nuclear stain (Fig. 3C).

After repeated OA inhalations, DNA synthesis was markedly increased in airway epithelial cells (Fig. 4A). A comparison of BrdU-positive epithelial cells among large, medium, and small airways in OA-treated animals was also statistically different (Fig. 4A). However, the effect of airway size disappeared after correction of the number of BrdU-positive epithelial cells for airway size (Fig. 4B and C). In response to antigen challenges, the number of airway epithelial cells that incorporated BrdU increased ~37-fold in large airways, 58-fold in medium airways, and 21-fold in small airways compared with those obtained from control animals (Fig. 4C). The labeling index, which is defined as the number of BrdU-labeled nuclei per total number of nuclei, for epithelial cells from large, medium, and small airways from antigen-challenged rats was $49.3 \times 10^{-2} \pm 1.2 \times 10^{-2}$, $58.8 \times 10^{-2} \pm 3.2 \times 10^{-2}$, and $37.8 \times 10^{-2} \pm 2.4 \times 10^{-2}$, respectively. The labeling index for epithelial cells from large, medium, and small airways from control rats was $1.7 \times 10^{-2}$, $1.3 \times 10^{-2}$, and $2.3 \times 10^{-2}$, respectively. The airway epithelium was normal on light microscopy.

Although repeated allergen inhalations also induced DNA synthesis in ASM cells, fewer airway myocytes

Fig. 4. OA challenge induced DNA synthesis in airway epithelial cells. A: quantitation of BrdU incorporation in airway epithelial cells. HPF, high-powered field. After repeated OA inhalations, number of airway epithelial cells incorporating BrdU increased compared with that obtained from Cont animals. Data are means \( \pm \) SE from 50 airways from 3 animals/group. Significant difference for OA-challenged vs. control animals, \( P < 0.001 \) by analysis of variance (Bonferroni-Dunn test). B: BrdU incorporation in airway epithelial cells standardized to airway size (1 \( \times \) 10\(^{-3} \) cells/mm basement membrane). In comparison with Cont animals, OA-treated animals had marked increases in BrdU-positive epithelial cells; \( P < 0.001 \) by analysis of variance (Bonferroni-Dunn test). However, there appears to be no difference between airway size and number of BrdU-positive epithelial cells in OA-treated animals. C: antigen inhalations markedly increased DNA synthesis in airway epithelial cells. Data are increases in BrdU incorporation over control values. There was no difference between airway size and number of BrdU-positive epithelial cells in OA-treated animals.
incorporated BrdU than did the airway epithelial cells (Figs. 4A and 5A). In a manner similar to that observed with epithelial cells, there were marked increases in the number of BrdU-positive ASM cells among large, medium, and small airways in OA-exposed animals compared with control saline-challenged animals (Fig. 5A). However, in contrast to OA-induced DNA synthesis in airway epithelial cells, after correction for airway size, marked differences in the number of BrdU-positive myocytes emerged (Fig. 5B). The number of BrdU-positive airway myocytes per square micrometer was greatest in the small airways, intermediate in the medium airways, and least in the large airways in OA-treated animals. However, in control animals, small differences also existed between airway size and the number of BrdU-positive airway myocytes. When the data were represented as increases over the control values, the number of ASM cells that incorporated BrdU increased ~35-fold in large airways, 19-fold in medium airways, and 9-fold in small airways (Fig. 5C). The labeling index for smooth muscle cells from large, medium, and small airways from antigen-challenged rats was $39.9 \times 10^{-2} \pm 1.3 \times 10^{-2}$, $23.4 \times 10^{-2} \pm 1.3 \times 10^{-2}$, and $21.6 \times 10^{-2} \pm 2.7 \times 10^{-2}$, respectively. The labeling index for epithelial cells from large, medium, and small airways from control rats was $1.3 \times 10^{-2}$, $1.3 \times 10^{-2}$, and $2.7 \times 10^{-2}$, respectively. The changes in BrdU incorporation were specific for the airways, and

Fig. 5. Repeated allergen challenge induced DNA synthesis in airway smooth muscle (ASM) cells. A: quantitation of BrdU incorporation in ASM cells. After repeated OA inhalations, number of airway myocytes incorporating BrdU increased compared with that obtained from Cont animals. Data are means ± SE from 50 airways from 3 animals/group. Significant difference for OA-challenged vs. control animals, $P < 0.001$ by analysis of variance (Bonferroni-Dunn test). B: BrdU incorporation in airway myocytes standardized to airway size ($1 \times 10^{-6}$ cells/mm basement membrane). In comparison with Cont animals, OA-treated animals had marked increases in BrdU-positive ASM cells, $P < 0.001$. Greatest increase in number of BrdU-positive ASM cells/mm was observed in small and medium airways and, to a lesser extent, in large airways. Significant difference for small vs. large or medium airways from OA-treated animals, $P < 0.01$. C: antigen inhalations increased DNA synthesis in airway myocytes. Data are increases in BrdU-positive ASM cells from OA-treated rats over those in Cont rats. In OA-treated animals, greatest increase in BrdU-positive ASM cells was observed in large and, to a lesser extent, medium airways compared with those in small airways.
no increases in the number of BrdU-positive vascular smooth muscle cells were seen in OA-exposed animals (data not shown).

Effects of OA challenge on morphometrically determined ASM. As shown in Fig. 6, there was a substantially greater quantity of ASM in the airways of OA-challenged animals \((n = 6)\) than in the saline-challenged control animals \((n = 6)\). The greatest difference was evident in the large airways where OA-challenged animals had seven times the quantity of muscle than the control animals. This difference was only twofold in medium-sized airways and disappeared entirely in the small airways.

**DISCUSSION**

The results of the present study confirm that allergen-induced remodeling of the airways occurs in the actively sensitized and OA-challenged BN rat. There was an increase in the morphometrically determined ASM mass. There was a gradient in ASM mass, standardized for airway size, from the large to the small airways. The difference in muscle was most striking in the large airways and absent in the smallest airways. There was also evidence of increased DNA synthesis by ASM cells after OA challenge, suggesting that a significant part, if not all, of the change in ASM mass may be attributable to hyperplasia. The discordance between the increased incorporation of BrdU into small-airway smooth muscle cells and the lack of any increase by morphometry is not explained. Interestingly, and somewhat unexpectedly, there was also an increased incorporation of BrdU into epithelial cells. This finding was not accompanied by any apparent morphological alteration of the epithelial cells on light microscopy.

Inhaled antigen challenges induced increased numbers of BrdU-positive cells throughout the tracheobronchial tree. Absolute numbers of BrdU-positive cells were greatest in the large conducting airways and decreased with the size of the airways. Similar effects on DNA synthesis in bronchial epithelial cells have been reported in rats exposed to inhaled tobacco smoke, sulfur dioxide, or nitrogen dioxide (see Ref. 1 for a review). These studies determined that the mitotic index of bronchial epithelial cells increased 10-fold in response to tobacco smoke \((25)\), 12-fold with sulfur dioxide \((15)\), and 50-fold with nitrogen dioxide compared with control animals \((8)\). Our results confirm that antigen inhalation induces similar increases in DNA synthesis in epithelial cells; however, our techniques could not discriminate among epithelial cell types. Further studies are needed not only to determine whether the growth response is restricted to specific types of airway epithelial cells but also to characterize whether proliferating epithelial cells in vivo manifest phenotypic changes that modulate their function. Such changes could, in themselves, have a bearing on ASM growth in view of the known epithelial products with potential growth-modulating effects \((9, 18)\). Prostaglandin \(E_1\) is a potent growth-retarding substance \((9)\), whereas endothelin causes thromboxane and leukotriene \((LT)\) \(C_4\) synthesis and mitogenesis in vitro \((18)\).

Our results confirm previous studies showing that repeated antigen inhalations increase bronchial ASM mass and extend these observations to demonstrate a marked effect of inhalational allergen challenge on DNA synthesis in ASM cells \((24)\). Although a recent report \((12)\) suggested that hyperoxia exposure in immature rats increased BrdU fractional labeling in ASM, few studies have examined ASM cell turnover in vivo. It was of interest that BrdU labeling of vascular smooth muscle and endothelium in the lungs of OA-sensitized and -challenged animals was minimal and similar in magnitude to that observed in control animals. These data suggested that the stimulatory growth effects on ASM and epithelium are likely dependent on local growth signals, distinct from mitogens that may act systemically.

The precise mechanisms that induce DNA synthesis in airway myocytes in vivo remain unknown. However, several growth factors and contractile agonists have recently been identified as ASM cell mitogens in vitro and, potentially, these agents may be important in airway remodeling after antigen challenge \((19)\). An LTD\(_4\) antagonist, MK-571, has been shown to prevent allergen-induced changes in ASM in the BN rat when administered before and shortly after OA challenge in a dose confirmed to have reduced early and abolished late responses \((24)\). The data are in accord with the previous demonstrations of the importance of cysteinyl-LT in allergic airway responses in the rat \((17)\). Whether LTD\(_4\) acts directly as a mitogen or indirectly through the release of more classic growth factors such as platelet-
derived growth factor is not known. LTC₄ has been shown to release fibroblast growth factor from macrophages in culture (21). In addition to soluble growth-promoting substances, other mechanisms that are dependent on direct cell-cell interactions may also be important in stimulating myocyte growth. Recently, our laboratory (16) reported that activated T lymphocytes, which adhere to ASM cells through intercellular adhesion molecule-lymphocyte function-associated antigen-1, vascular cell adhesion molecule-very late activating antigen-4, and CD44-hyaluronate interactions, induce ASM cell proliferation. Because antigen inhalation likely activates resident T lymphocytes as well as recruits these cells into the bronchial submucosa, it is plausible that T lymphocyte-ASM cell interactions may play a role in stimulating ASM cell growth in vivo.

Although both an increase in ASM mass and BrdU incorporation occurred after allergen challenge, there was not complete concordance between the result of the two forms of analysis. No increase in ASM was measured in the small airways by morphometry. Despite that, BrdU incorporation was evident (4, 5). There are previous reports of dissociation of these phenomena. Although BrdU incorporation into DNA occurs as cells traverse the S phase of the cell cycle, BrdU labeling of DNA can also occur under other circumstances that may not correlate with cell proliferation. In cultured rat alveolar type II cells, it has been reported that [³H]thymidine incorporation occurs without cell proliferation and is the result of an unusual form of growth arrest (4). In another study (5), BrdU is incorporated at excision-repair sites of DNA in fibroblasts exposed to high doses of radiation. Although DNA synthesis without cell replication has been reported to occur in vitro, there is no evidence that this phenomenon occurs in any airway cell type in vivo. Our findings that antigen inhalation induced both increases in ASM mass and increases in the number of BrdU-positive myocytes lend support to the contention that the changes in ASM mass were due, at least in part, to myocyte hyperplasia. Because the marked increases in BrdU-positive airway myocytes in the small airways were associated with no significant effect on ASM mass, other mechanisms, perhaps myocyte apoptosis, may play a role in modulating ASM mass in vivo. The other issue that requires consideration is that of the size correction employed for the calculations of BrdU and ASM mass. We corrected for the size of the airway lumen in its ideal unconstricted state, assuming that it is a perfect circle. It is possible that this method of correction may overcorrect for size. Interestingly, the increases in muscle and BrdU incorporation as a function of airway size were less discordant, suggesting that standardization of BrdU incorporation for airway wall area may not be the most appropriate size correction. However, it appears that the corrected ASM area is similar in all sized airways in control animals, suggesting that indeed the correction is appropriate. Irrespective of such corrections, the data do not suggest that hypertrophy contributed to the changes in mass in that BrdU incorporation was demonstrable in all sized airways even when the mass of ASM was not measurably increased.

In summary, we confirmed that ASM mass was increased in the bronchi of actively sensitized BN rats after antigen challenge. Our results showed that changes in bronchial smooth muscle mass were associated with the stimulation of DNA synthesis in airway myocytes. There was also evidence for increased epithelial turnover despite a normal appearance of the epithelium on light microscopy. This model, therefore, may be useful in studying mechanisms that modulate ASM cell hyperplasia and airway epithelial cell growth after inhalational antigen challenges.

We thank Rosa Pantano for excellent technical assistance and Mary McNickel for diligence in preparing this manuscript. R. A. Panettieri, Jr., was supported by National Heart, Lung, and Blood Institute Grants HL-02647 and ROI-HL-55301; National Aeronautics and Space Administration Grant NRA-94-OLMSA-02; a Career Investigator Award from the American Lung Association; the Pennsylvania and National Lung Associations; the University Research Foundation; and SmithKline Beecham. J. G. Martin was supported by Grant MA-7852 from the Medical Research Council of Canada.

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Received 30 July 1997; accepted in final form 25 November 1997.

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