Salicylate effects on a monolayer culture of gastric mucous cells from adult rats

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SUMMARY   Aspirin, acetyl salicylic acid, damages gastric mucosal cells. This effect is considered related to its inhibition of prostaglandin synthesis. On the other hand, sodium salicylate has been reported to be cytoprotective against drug damage to gastric mucosa *in vivo*.[1][2] One reason for this difference is that salicylic acid, unlike acetyl salicylic acid does not inhibit prostaglandin synthesis by gastric mucosa *in vivo*. Previous studies on tissue culture cells from our laboratory have required gastric mucosa from fetal rats; this was time consuming and expensive. The purpose of this study was to develop a primary cell culture of adult rat fundic epithelial cells and to test the effect of sodium salicylate on: (1) prostaglandin (PGE_2) production, (2) cell viability, (3) reducing cell damage by sodium taurocholate. Gastric epithelial cells were isolated from adult rat stomachs and cultured on collagen gel. Cells reached confluency on day 4 at which stage fibroblasts were rarely seen (<1%). Autoradiographic study showed that cultured cells incorporated [3-H] thymidine into nuclei. In histochemical studies, 94% of the cells contained PAS positive granules (mucous cells). Mucous granules were observed in the cytoplasm of the majority of cells by electronmicroscopy. These cells synthesised prostaglandin E_2 as determined by radioimmunoassay. Indomethacin 10^{-4} M strongly suppressed PGE_2 production after 30 minutes while 10^{-3} and 10^{-4} M sodium salicylate had no effect. Pharmacologic concentrations of 10 mM sodium salicylate had no effect on PGE_2 production at 30 minutes and only weakly inhibited production after one hour incubation. Sodium salicylate up to 30 mM had no effect on cell viability, a concentration of 50 mM being necessary to produce significant cell damage. Sodium salicylate 10 mM significantly protected cells against damage induced by 10 mM sodium taurocholate. We conclude: (i) adult rat gastric mucous epithelial cells can be successfully cultured *in vitro*; (ii) prostaglandin synthesis is inhibited by indomethacin but not by low doses of sodium salicylate; (iii) sodium salicylate does not damage gastric mucosal cells except at very high concentrations; (iv) sodium salicylate protects against damage to cells induced by sodium taurocholate.

Sodium (Na) salicylate has been reported to protect gastric mucosa against damage by aspirin, ethanol and concentrated hydrochloric acid *in vivo*.[1][2] Stimulation of endogenous prostaglandin I_2-like activity was associated with this cytoprotection,[1] but blockage of prostaglandin synthesis did not prevent

such protection making it unlikely that this was the mechanism of protection.[2]

Cell culture of gastric epithelial cells is a valuable model for the study of cell function without systemic factors. Successful culture of gastric epithelial cells[3-5] has been reported but in order to establish cell growth and to avoid contamination, fetal tissue has been used in most cases. Soll *et al* reported a monolayer preparation of adult canine fundic mucosa which was mainly composed of chief cells.[6] In

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their preparation, cell division was not observed. Furthermore, in order to establish cell growth in theirs and other culture systems, addition of hormonal factors was required which in itself, may alter the functional status of cultured cells.

As the *in vivo* studies with Na salicylate were done in adult rats,[12] cultured cells from adult rats may be expected to more accurately reflect functions of adult gastric cells. Accordingly, we modified our established techniques for culture of baby rat gastric cell culture[7] in order to culture adult rat gastric cells.

The morphologic characteristics of these cells, their ability to divide and to synthesise prostaglandins and the effect of sodium salicylate and indomethacin on prostaglandin synthesis were studied. In addition, the effect of sodium salicylate on cell viability and its ability to exert a cytoprotective effect *in vitro* were studied.

Methods

MATERIALS

Medium I: Coon's modified Ham's F-12 medium[8] (F-12 medium, KC Biological Inc, Lenexa, KS) containing 0·15 g/dl collagenase (120 U/mg, GIBCO, Grand Island, NY), 100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml fungizone (these antibiotics and antimycotic (AA) were obtained from Irvine Scientific Co, Santa Ana, CA). Medium II: F-12 medium supplemented with heat inactivated (at 56°C for 30 minutes) 10 g/dl fetal bovine serum (Armour Pharmaceutical Co, Kankakee, IL), 15 mM Hepes buffer (Sigma Chemical Co, St Louis, MO), fibronectin (10 µg/ml; Sigma) and AA described above. Trypsin was purchased from Sigma. [3-H] thymidine ([3-H]Tdr, specific activity 20 Ci/mmol), (125-I) prostaglandin E_2 (PGE_2) and 6-Keto-PGF_{1a} (6KF) radioimmunoassay (RIA) kits were purchased from New England Nuclear, Boston, MA. Nuclear Track Emulsion (NBT-2), D19 and Rapid Fix were obtained from Eastman-Kodak, Rochester, NY.

CELL PREPARATION

The stomachs from rats (Sprague-Dawley Albino, Simonsen Lab, Gilroy, CA) weighing 250–350 g were placed in Hank's balanced salt solution (HBSS). The stomachs were opened along the lesser curvature and the fundic area was excised. The fundic musoca was washed vigorously with three changes of HBSS. The mucosa was incubated in 20 ml of medium I at 37°C in a shaking water bath for 60 minutes. The medium was discarded and mucosa incubated in 20 ml fresh medium I for an additional 15 minutes. This medium was discarded and the mucosa was manually shaken for two minutes in 20 ml fresh medium I. The mucosa was discarded and the cell suspension finally incu-

bated for 20 minutes at 37°C. The cell suspension was then pipetted several times and filtered through 200 µmol sterile nylon mesh (Nytex, Tetko, Elmsford, NY). Four millilitres of the filtrate, containing cell clumps, was overlaid on 10 ml of 0·5 g/dl bovine serum albumin (BSA, Sigma) containing F-12 medium and centrifuged at 600 rpm for five minutes. The pellet was washed three times with F-12 medium containing AA. The washed pellet was resuspended in medium II. Sterile technique was used throughout these procedures.

COLLAGEN GEL PREPARATION

Collagen was prepared from rat tails. One gram of the tendons of rat tails was dissolved in 300 ml 1:1000 acetic acid solution. The solution was stored at 4°C for 48 h and then centrifuged at 2500 g at 4°C for 20 minutes. The supernate was stored at 4°C until dialysis. The solution was dialysed for 48 h using dialyser tubing (Fisher Scientific, Pittsburgh, PA). 0·5 ml of dialysed solution was added to a 24 multiwell culture dish (Falcon, Oxnard, CA), and then gelled by exposure to ammonia vapour. Dishes were kept under ultraviolet light. Dishes were incubated with medium II two hours before use.

CULTURE

Medium II, containing cells, was inoculated onto the culture dish prepared as described above. About 3×10^5 cells in 0·5 ml medium was inoculated into one well. The cultures were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2 in air. The media was changed daily.

MORPHOLOGICAL STUDIES

Cultures were examined daily with a phase contrast microscope. Cells were stained with Giemsa solution on each day. A mitotic index was calculated. The trypan blue dye exclusion test for viability of isolated cells was carried out by the method of Phillips,[9] as previously described.[7]

HISTOCHEMICAL STUDIES

To identify parietal cells succinic dehydrogenase activity was determined by the method of Nachlas *et al.*[10] The cultured cells were incubated in the medium containing 0·2 M phosphate buffer (pH 7·6), 0·2 M succinic acid (disodium salt), and Nitro-BT (1 mg/ml, Sigma) for 60 minutes at 37°C. Cells were counterstained by 1% saffranin. To distinguish mucous cells both periodic Acid-Schiff (PAS) and alcian blue (AB) staining were used. Cells were fixed with buffered formalin and stained with 1% alcian blue staining solution for 30 minutes. Cells were then also stained with PAS reagent. PAS staining alone was also performed. To stain chief cells, Bowie Stain was
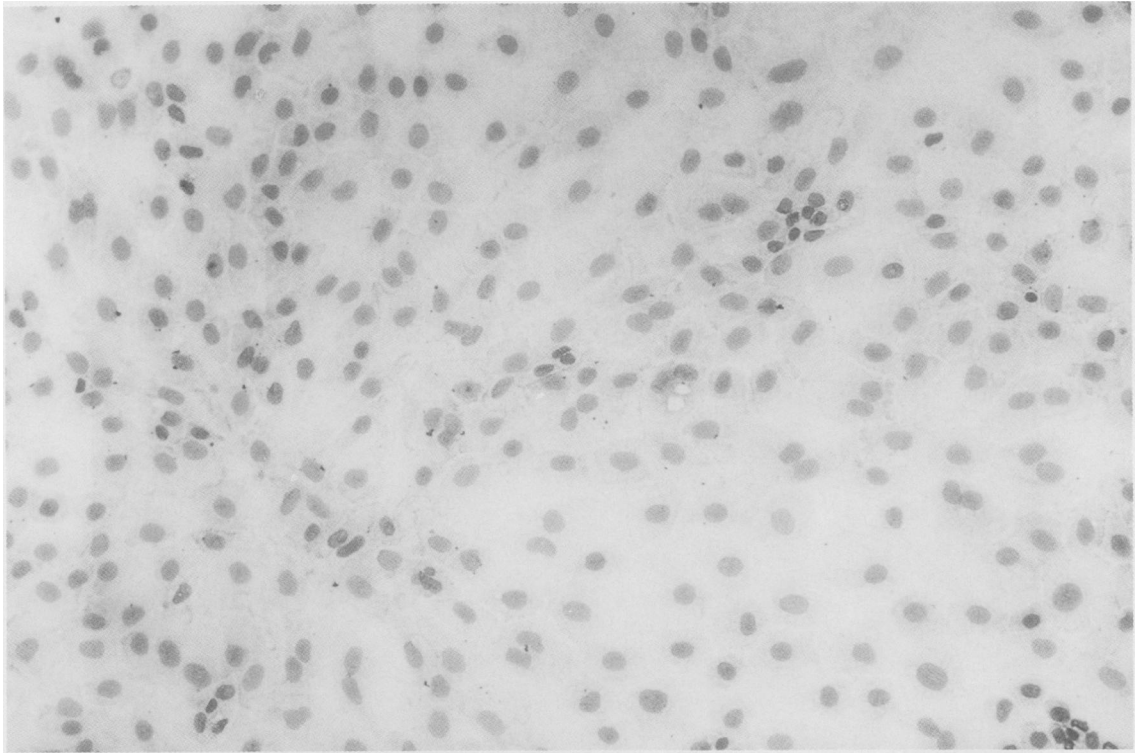
Fig. 1 *Giemsa-stained adult rat gastric epithelial cells in tissue culture. Four day old culture. With this stain only cell nuclei stain prominently.*

used.[11] Neutral dye was prepared by mixing Orange G and crystal violet staining solution. Cells were stained with neutral dye solution for 10 minutes. These staining techniques were applied to gastric mucosa, isolated cell clumps, and cultured cells.

ELECTRON MICROSCOPY
Monolayers were fixed with 2% glutaraldehyde for 20 minutes and postfixed with 1% OsO_4 for 20 minutes. The cells were dehydrated with graded alcohol and embedded in Epon 812 and polymerised at 60°C overnight. The sections were stained with uranyl acetate followed by lead citrate.

CELL GROWTH
The number of cells in each well was counted each day using the nuclear counting method.[12] Cultured cells were dislodged by incubation of the cells in 0·25% trypsin for 10 minutes. The cells were then incubated in 0·1 M citric acid for one hour at 37°C and pipetted vigorously. The nuclei were collected by centrifugation at 2000 rpm for 20 minutes and suspended in the 0·1% crystal violet solution. The

nuclei were counted using a haemacytometer. The number of cells after isolation was also counted as described above.

AUTORADIOGRAPHY
Autoradiography was done using a modification of the procedure of Hamlin and Pardee.[13] The cells were incubated with 1 μCi/ml [3-H]Tdr for six hours each day. Cells were fixed with methanol and dried. NBT-2 emulsion was added to the cells. The cultures were stored in the dark at 4°C for one week. They were developed in Kodak D19 for six minutes and fixed in Kodak Rapid Fix for three minutes. The cells were poststained with haematoxylin. Labelling index was expressed as percentage of the labelled nuclei per 100 cells.

PROSTAGLANDIN PRODUCTION BY CULTURED GASTRIC CELLS
Media contents of PGE_2 and 6KF were measured by radioimmunoassay (RIA). Monolayers were washed three times with F-12 medium (37°C) and incubated with medium for 30 minutes for further washing.
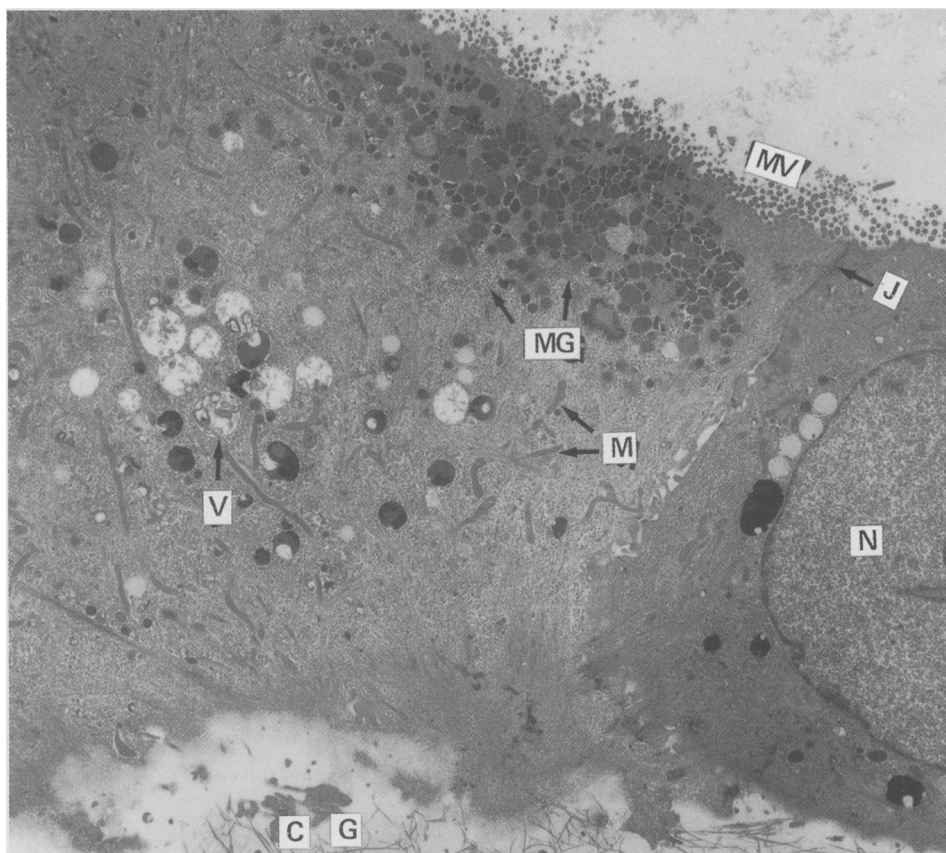
Fig. 2   *Transmission electron micrograph of adult rat gastric cells in tissue culture, four day old culture showing cytoplasm of adjacent cells and tight junctions between cell membranes. N, nucleus; MV, microvilli; m, mitochondria; MG, mucous granules; V, autophagic vacuoles; J, junctional complex.*

This medium was discarded and monolayers were incubated with medium containing test reagents or medium only for 60 minutes. The medium was collected and centrifuged for five minutes at 2000 rpm and 4°C. The supernatant was collected and stored at −20°C until assayed. The pellet was combined with the corresponding monolayer and protein was determined with the dye binding assay according to Bradford.[14] PGE_2 and 6KF content of media were assayed in duplicate with highly sensitive PGE_2 and 6KF 125-I RIA kits. Standards in the range of 0·25 to 50 pg PGE_2 and 2 to 50 pg 6KF were treated in the same way as the samples and the curve calculated on semi-logarithmic paper after counting in a Beckman 7000 gamma counter. The limits of detection were 0·2 pg PGE_2 and 2·0 pg 6KF per assay tube. For PGE_2, cross-reactivity with PGE_1 was 3·7% and with all other prostaglandins less than 0·4%. For 6KF, cross-reactivity with PGF-2a was 2·6% and for all other prostaglandins less than 2%

EXPERIMENTAL DESIGN

Effect of Na salicylate on cell viability
Cultured cells were incubated for one hour with: (a) medium only (control), (b) Na salicylate 0·1 mM, (c) Na salicylate 1 mM, (d) Na salicylate 10 mM, (e) Na salicylate 30 mM, (f) Na salicylate 50 mM. After incubation, cells were washed with medium and kept in 1 ml of F-12 medium. 0·1 ml trypan blue (0·4%) was added to the medium. The number of stained or non-stained cells were counted and the percentage of viable cells was expressed as non-stained cells/(stained+non-stained cells)×100.
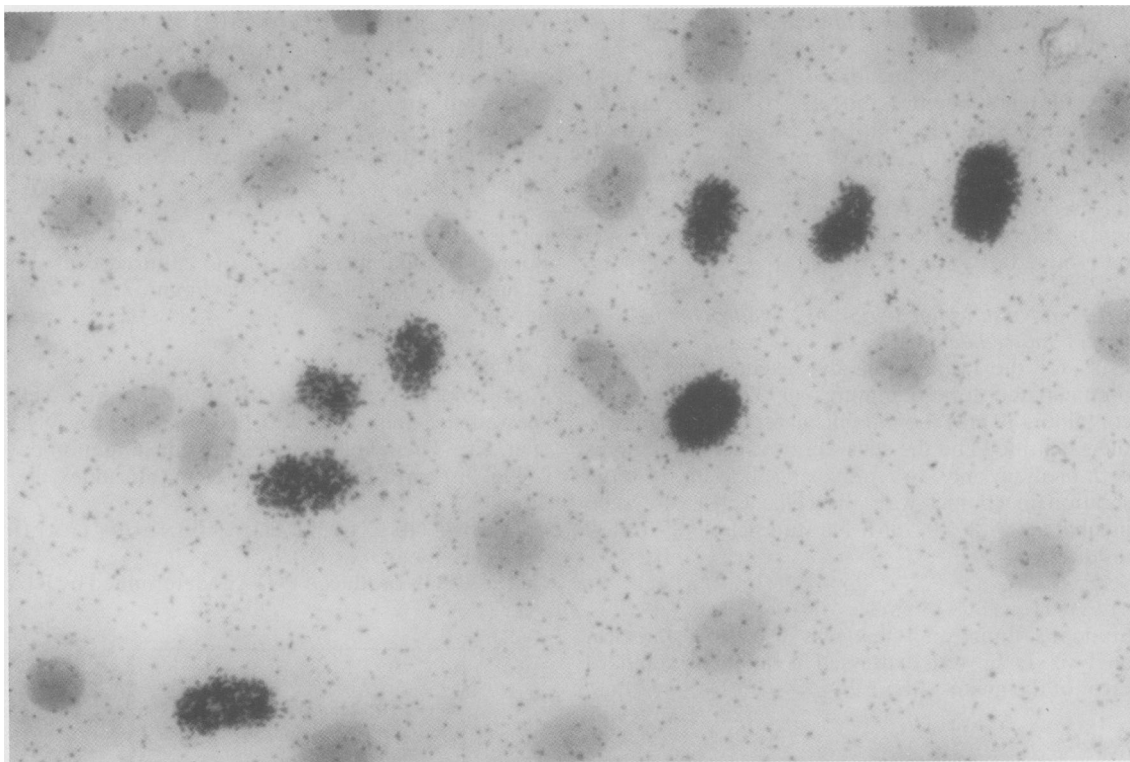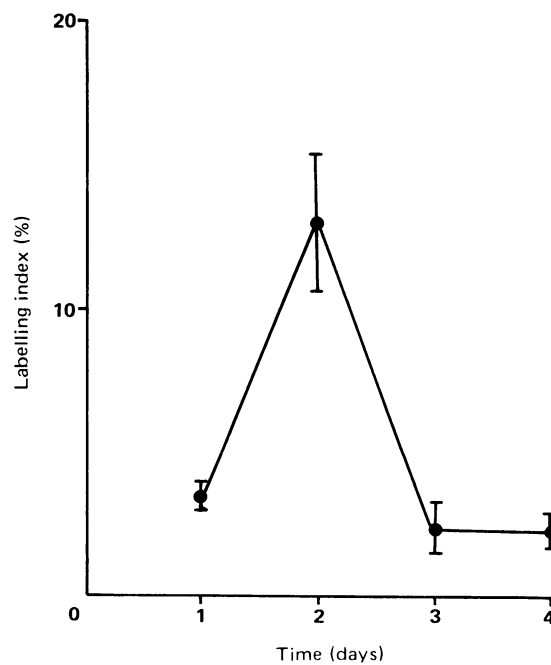
Fig. 3(a)

Fig. 3(b)

Fig. 3(a)   *Autoradiograph of [3H]thymidine incorporation in rat gastric epithelial cells in tissue culture (haematoxylin stain). Cells which contained more than 10 silver grains in the nucleus were considered as labelled cells.*[7]

Fig. 3(b)   *Labelling indices determined by autoradiography of [3H]thymidine. Each point represents the mean (SE) of four cultures.*

Effect of Na Salicylate and indomethacin on the production of PGE$_2$

Cultured cells were incubated for nil to one hour with: (a) medium only (control), (b) Na salicylate 0·1 mM, (c) Na salicylate 1 mM, (d) Na salicylate 10 mM, (e) indomethacin 10^{-4} M. After incubation, medium was collected and stored at −20°C. PGE$_2$ content of the medium was assayed directly. Cells were trypsinised, collected by centrifugation and protein was determined.[14]

Effect of Na salicylate (NaS) on Na taurocholate (NaT) induced damage

Cells for the trypan blue dye exclusion test were preincubated for 30 minutes at 37°C in medium containing 10 mM sodium salicylate; the medium was then aspirated and the cells were washed three times with medium only, to remove all salicylate from the solution in contact with the cells; cells were then incubated with 10 mM Na taurocholate for 30 minutes.

STATISTICAL ANALYSIS

Student's unpaired *t* test was used for statistical analysis. Data were expressed as the mean standard error of the means (SE).

Results

LIGHT MICROSCOPY

Cell isolation: the cells from the final incubation were mainly composed of cell clumps and showed good viability (93±3%).

Cell culture: within one day after inoculation, the cell clumps attached to the surface of the collagen gel. The attachment of cells was markedly increased by use of collagen gel and by addition of fibronectin to the cell media. Cells began to grow and reached confluency on day 4 (Fig. 1). Over 99% of the cells at this stage had epithelial characteristics with only 0–1% fibroblasts. In most cultures by day 6 or day 7 fibroblasts were seen and their outgrowth started. Bacterial or fungal contamination was not observed up to two weeks.

HISTOCHEMICAL STUDIES

In the clumps after isolation of surface mucous cells, mucous neck cells, parietal cells and chief cells were observed. In four day old cultures, 94% of the cells contained PAS positive material in the cytoplasm; identifying them as mucous cells; 3% of the cells showed a strong reaction for succinic dehydrogenase activity consistent with parietal cells. Bowie staining for pepsinogen cells was negative; 3% of the cells were epithelial type cells which were unidentifiable histochemically.

TRANSMISSION ELECTRON MICROSCOPY

Ultrastructurally, microvillus like projections were apparent on the surface of the cells. Cells were connected by junctional complexes with tight junctions and desmosomes. The majority of the cells had electron dense granules characteristic of mucous granules (Fig. 2).

CELL PROLIFERATION

Population doubling time of the cultured cells was 22 hours. Mitotic index was maximum, 2·2±2%, on day 2.

DNA SYNTHESIS

Autoradiography of [3H]TdR in the cultured cells showed the ability of the cells to synthesise DNA (Fig. 3a). The labelling index was maximum on day 2 (Fig. 3b) corresponding with the mitotic index.

EFFECT OF Na SALICYLATE AND INDOMETHACIN ON PGE-2 PRODUCTION
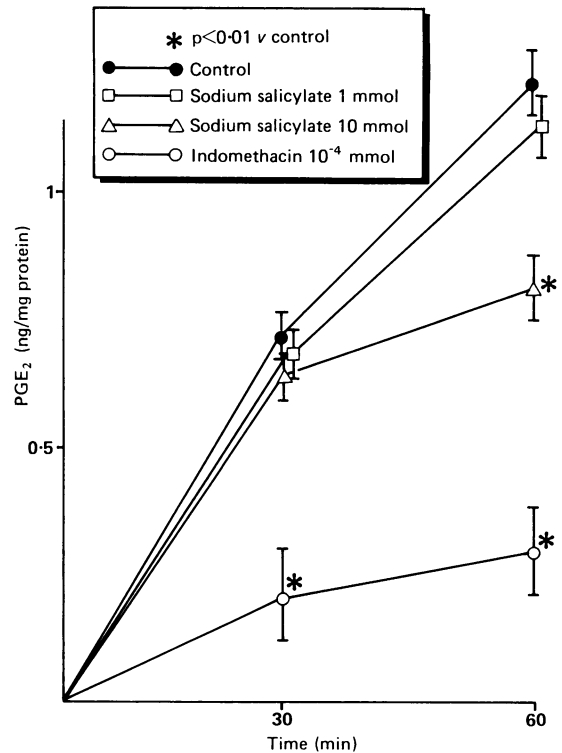
Cultured cells produced PGE$_2$ as determined by RIA

Fig. 4 *The effect of sodium salicylate and indomethacin on the production of PGE$_2$. Each point represents mean (SE) of five to six cultures. Asterisks indicate statistically significant change from the control value (*p<0·01).*
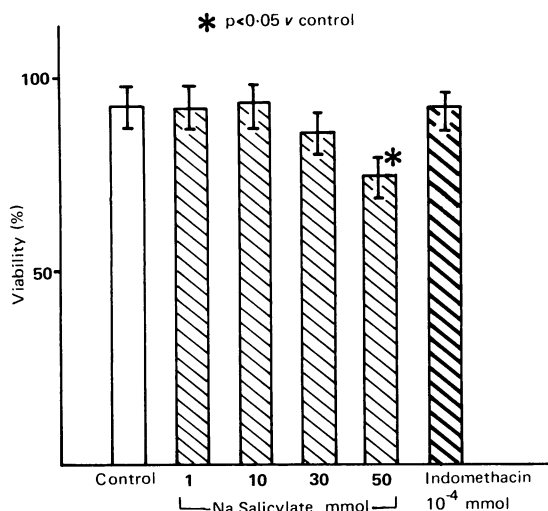
Fig. 5 *Effect of sodium salicylate on cell viability. Each point represents mean (SE) of five to six cultures. Asterisk indicates statistically significant change from the control value (*p<0·05).*

Fig. 6 *Effect of sodium taurocholate (NaT) on cell viability. Protective effect of 10 mM sodium salicylate (NaS).*

$(1·21$ ng/mg protein/h). One millimole $(10^{-3}$ M) and $0·1$ mM $(10^{-4}$ M) Na salicylate had no effect on PGE_2 production (Fig. 4). After one hour's incubation 10 mM $(10^{-2}$ M) Na salicylate weakly but significantly inhibited PGE_2 production while 10^{-4} M indomethacin strongly suppressed PGE_2 production even at 30 minutes.

EFFECT OF Na SALICYLATE ON CELL VIABILITY
After 60 minutes incubation, cultured cells showed good viability $(92\pm7\%)$. Na salicylate up to a concentration of 30 mM did not damage gastric mucosal cells. A concentration of 50 mM Na salicylate was necessary to damage the cultured cells significantly. Indomethacin 10^{-4} M did not have any effect on cell viability (Fig. 5).

EFFECT OF Na TAUROCHOLATE ON CELL VIABILITY
Na Taurocholate at 0 mM and 2·5 mM had no effect on cell viability; at 5 mM and 10 mM concentrations of Na taurocholate viability was reduced to $67\pm5\%$ and $34\pm6\%$ respectively (Fig. 6).

EFFECT OF Na SALICYLATE ON Na TAUROCHOLATE INDUCED CELL DAMAGE
After preincubation with 10 mM Na salicylate followed by removal of the Na S, subsequent exposure to Na T 10 mM resulted in $57\pm2\%$ viability compared with $34\pm6\%$ without Na S $(p<0·01)$ (Fig. 6).
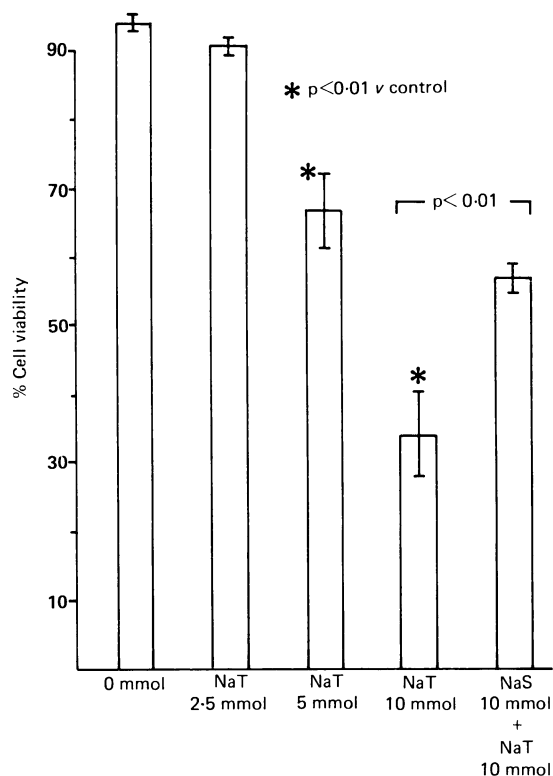
Discussion

We have developed a monolayer culture of adult rat fundic epithelial cells. A major factor in the successful growth of adult cells in tissue culture as opposed to fetal cells was the use of collagen gel as a substrate to encourage the isolated adult cells to attach to the culture dishes. Monolayer culture systems of gastric mucosa have been reported using newborn rat gastric mucosa,[7] fetal rabbit gastric mucosa,[3 4] adult canine gastric mucosa,[6] and adult human gastric mucosa.[3 15] In the present study, adult rat gastric mucosa was used which offers several advantages over fetal tissue culture cells: as adult rats are commonly used in most laboratories for *in vivo* studies, cultured cells from adult rats may more accurately reflect the physiological condition of adult gastric mucosa than fetal cells; adult rats are less expensive and less time consuming to obtain than are fetal rats from breeding colonies; experimental schedules consequently are much easier to arrange.

Fibroblast overgrowth has been a major problem of epithelial cell culture and various attempts to eliminate such contamination have been

reported.[16-19] In our previous report using newborn rats,[7] about 5% fibroblasts were observed at the confluent stage (day 3). In the present study, less than 1% fibroblasts existed in culture up to day 5 and fibroblast overgrowth started much later. These findings are similar to those we reported with adult human gastric cell culture[15] which was also relatively fibroblast free possibly because biopsy specimens contained very small numbers of fibroblasts thus fibroblast overgrowth could be avoided. Because the gastric mucosa of adult rats is thicker than that of baby rats, fewer fibroblasts may be obtained in the isolation process; alternatively, fibroblasts from adult rats may grow more slowly than those of baby rats.

The ability of the culture cells to proliferate and to synthesise DNA was shown by study of mitosis, incorporation of [3H]TdR into cellular nuclei, and cell growth. Most of the cells in this culture system were mucous cells. This indicates that of the mixed epithelial cells inoculated into the culture – that is, mucous cells, parietal cells, and chief cells, only the mucous cells were able to divide and replicate indicating that these cells were most likely mucous neck cells. Tissue cultures of mucous cells are particularly apt for studies of drug damage. In vivo in man, drug damage with common agents such as aspirin predominantly involves damage to the lining surface mucous cells.[20] More severe necrotic damage as seen with experimental irritants such as absolute alcohol in animals is associated with concomitant ischaemic damage to the deeper layers of the mucosa;[21] however, repair of this damage is dependent on the regenerative ability of the mucous (neck) cells.[22 23]

The cultured mucous cells produced PGE_2. In a previous study, we have shown that such mucous cells produce more PGE_2 than PGI_2 in the order of 4:1.[24] Konturek et al[1] reported that Na salicylate increased PGI_2-like activity by gastric mucosa in an in vivo rat study and attributed the cytoprotective effect of Na salicylate to this stimulation. In an in vivo study by Robert, however, Na salicylate induced cytoprotection which was not blocked by indomethacin, so that salicylate cytoprotection could not be attributed to prostaglandin production.[2] Whittle et al in an ex vivo study reported that sodium salicylate inhibited prostaglandin production in subcutaneous inflammatory lesions but not in gastric mucosal tissue.[25] In an in vitro study of non-gastric cells, Na salicylate was found to be a weak cyclooxygenase inhibitor.[26] The current studies are in agreement with these studies[2 25 26] and indicate that Na salicylate either has no effect on, or in pharmacological doses is a weak inhibitor of prostaglandin E_2 production by gastric mucous cells. These data also suggest that Na

salicylate cytoprotection of gastric mucous cells is unlikely to be related to stimulation of PGE_2 production by these cells.

Sodium salicylate was not toxic to cells except in the very high concentration of 50 mM. These results are similar to findings of lack of toxicity of sodium acetylsalicylate to tissue culture cells at neutral pH, 7·4.[27] Prostaglandin values were not measured after administration of damaging doses of sodium salicylate (50 mM) or taurocholate. Cell death and lysis is itself associated with increased prostaglandin release, a non-specific effect.[1]

The protective effect of sodium salicylate on sodium taurocholate induced toxicity is consistent with its cytoprotective effects in vivo[1] and shows that such effects can also occur in vitro in the absence of systemic factors. These results are in agreement with our previous findings that the analgesic acetaminophen, which like salicylic acid is protective against drug damage to gastric mucosa in vivo,[1] is also cytoprotective in vitro. Furthermore, acetaminophen had no effect on PGE_2 or PGI_2 production in vitro and its cytoprotective effect in vitro was not blocked by indomethacin, although indomethacin significantly inhibited PGE_2 and PGI_2 production.[28 29]

The conditions of these in vitro studies may be considered less physiological than in vivo studies in the sense that it is possible that both sides of the cells could be bathed in the media containing damaging agents. Nevertheless, in this and previous studies with varying agents cytoprotection could be demonstrated.[27-33] Indeed, cytoprotection of human isolated gastric glands by prostaglandins has recently been shown in spite of the cells being bathed on all sides by indomethacin or ethanol solutions.[34] Further, these authors found a correlation between the degree of protection in vitro of that found in their own in vivo studies.[35] This indicates that cytoprotection is a function of the cell as a whole and is not dependent on which surface of the cell is exposed or on cell polarity.

Because these protected cells are in vitro, protection against taurocholate induced damage must include the direct effect of sodium salicylate on cell membranes or metabolism. Physiologically, taurocholic acid has a pKa of <2. Therefore at pH 7·4, it is entirely in the dissociated state with no lipid solubility or ability to penetrate the cell. At this pH its damaging action is limited to a detergent action on the external cell membrane. Salicylate affects the barrier function of the external cell membrane as indicated by preventing trypan blue from penetrating the cell; this suggests that the cell membrane is the site of the protective action of salicylate.

In summary, we have described a method for successful monolayer culture of adult rat gastric mucous epithelial cells. PGE_2 production by these

cells was inhibited by indomethacin but only weakly by sodium salicylate even at pharmacological concentrations. Sodium taurocholate damaged gastric cells most likely by a detergent type action on the external cell membrane. Sodium salicylate at neutral pH was not damaging to cells except at very high concentrations. Sodium salicylate was protective against sodium taurocholate induced damage to gastric mucosal cells *in vitro* independent of systemic factors. This direct protective effect appears to be on the external cell membrane and is unlikely to be mediated by increased cellular metabolism of prostaglandins.

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