Protein transport and processing by human HT29-19A intestinal cells: effect of interferon γ

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Abstract

***Background*—The nature of the breakdown products produced in enterocytes during epithelial transport of intact proteins may be critical in determining the functional consequences of protein absorption.**

***Aim*—(*a*) To measure the transepithelial transport of horseradish peroxidase (HRP) and to identify the nature of HRP breakdown products released on the basal side of enterocytes and (*b*) to assess the role of interferon γ (IFNγ) on HRP transport and processing.**

***Methods*—HT29-19A intestinal cells were used to assess transepithelial transport of HRP in Ussing chambers, and the nature of breakdown products in the basal compartment was analysed by high performance liquid chromatography (HPLC).**

***Results*—(1) In control conditions, [^{3}H]HRP equivalent fluxes (3135 (219) ng/h per cm^2; mean (SEM)) comprised 50% amino acids, 40% peptides, and 10% intact HRP. Steric exclusion HPLC of the breakdown products indicated a wide range of molecular masses including a major peptide of about 1150 Da. Lysosomal aspartyl and thiol proteases were expressed but no HLA-DR surface expression was noted. (2) At 48 to 72 hours after IFNγ stimulation, [^{3}H]HRP equivalent fluxes increased significantly (7392 (1433) ng/h per cm^2) without modification of the relative proportions of amino acids, peptides, and intact HRP, and without modification of the distribution of breakdown products in HPLC. Lysosomal protease activities were not modified by IFNγ but HLA-DR expression was increased.**

***Conclusion*—Intestinal cells are able to process HRP into peptides potentially capable of stimulating the immune system. IFNγ stimulates the transport and processing of HRP thus increasing the antigenic load in the intestinal mucosa.**

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Keywords: enterocyte; transcytosis; macromolecular degradation; HPLC; mucosal immunity

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Antigen processing and presentation play a central role in regulating immune responses.[1] Although enterocytes are not conventional antigen presenting cells, they probably play a role, not only in antigen transport, but also in antigen presentation to underlying lymphocytes.[2–4] This process generally leads to oral tolerance, described as the down-regulation of the systemic immune response to orally administered antigens via the generation of active cellular suppression or clonal anergy.[5]

In antigen presenting cells, exogenous proteins are taken up by endocytosis and processed by cathepsins into peptides of 13 to 17 amino acids able to bind to MHC class II molecules. The MHC II/peptide complex is translocated to the external membrane for direct presentation to lymphocytes.[6]

The enterocyte is able to take up intact food proteins that have escaped luminal hydrolysis and transport them to underlying tissues. Although the capacity of enterocytes to process antigens as efficiently as conventional antigen presenting cells has been questioned,[4] transepithelial transport studies using the tritiated macromolecular tracer horseradish peroxidase (HRP) allowed us to show that a major degradative pathway was involved, leading to intracellular degradation.[7] The nature of the degradation products is crucial to the functional significance of this degradative pathway. Normally, proteins endocytosed by the enterocytes can be degraded into amino acids without any consequence to the immune system, or into peptides, some of which have functional/immune activity.

Proinflammatory cytokines such as interferon γ (IFNγ) are secreted by lymphocytes in the intestinal mucosa in pathological conditions such as Crohn's disease.[8] In these conditions, enterocytes up-regulate both MHC class II molecule expression[9 10] and epithelial permeability to small molecules[11 12] and macromolecules.[13 14] The expression of MHC or other restriction molecules on enterocytes may interfere with the nature and/or quantity of the antigen derived peptides formed during transepithelial transport. Another question asked in the present study was whether the stimulation of MHC class II expression in enterocytes also stimulates the processing of exogenous proteins into peptides that are susceptible to binding to known restriction molecules.

The aims of the present study were to investigate the capacity of enterocytes to generate peptides from an exogenous antigen, HRP, and to assess the effect of IFNγ on HRP transepithelial transport and processing, using the Transwell grown human intestinal cell line HT29-19A as a model to study epithelial function.

Methods

REAGENTS

Recombinant human IFNγ (rhIFNγ, specific activity 10^7 U/mg protein) was from TEBU (Le Perray en Yvelines, France), and HRP (type VI),

N-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide (substrate S2160), and bovine haemoglobin were from Sigma (Saint-Quentin Fallavier, France). [^{3}H]HRP (specific radioactivity 20 Ci/mmol) was generated and stored as previously described.[7]

Monoclonal antibodies (mAbs) directed against HLA-DR (clone L243) were from Becton Dickinson (Mountain View, CA, USA).

HUMAN HT29-19A INTESTINAL CELL LINE

The human colonic intestinal cell line HT29-19A, cloned from HT29 by Augeron *et al*,[15] was used. Between passages, intestinal cells were cultured at 37°C in Dulbecco's modified Eagle medium (Eurobio, Les Ulis, France) containing 10% heat inactivated fetal calf serum, 4 mM glutamine, and 50 µg/ml gentamicin, in a humidified atmosphere of 5% CO_2/95% air. The medium was changed daily and cells were passaged every week. For experimental purposes, cells were seeded at a density of 0.8×10^6 cells/cm^2 on polycarbonate Transwell permeable filters with a pore diameter of 0.4 µm and a surface area of 1.13 or 4.7 cm^2 (Costar, Brumath, France). Cells formed a confluent monolayer that reached stable electrical resistance of about 150 ohms/cm^2 after 21 days and were used for transport and processing studies from that time.

In some experiments, confluent cell monolayers were treated for 48 to 72 hours with rhIFNγ (100 U/ml), placed in either the basal or apical compartment of the Transwell system, to induce MHC class II molecule expression before transepithelial transport studies.

[^{3}H]HRP TRANSPORT ACROSS HT29-19A INTESTINAL CELL MONOLAYERS IN USSING CHAMBERS

IFNγ treated HT29-19A intestinal cells and untreated cells were cultured in Transwell culture plates, and mounted in small Ussing chambers with an exposed area of 0.15 cm^2. Cells were bathed on each side with 1.2 ml oxygenated thermostated Ringer solution and short circuited using an automatic voltage clamp (WPI, Aston, UK), after appropriate correction for fluid and system resistance. Electrical parameters including potential difference, short circuit current, and total ionic conductance were recorded at regular intervals during the two hour period of experimentation. A change in ionic conductance was considered an index of altered paracellular permeability. After equilibration, permeability markers were added to the apical compartment. HRP was added at a final concentration of 0.4 mg/ml (10 µmol/l), with a tracer dose of [^{3}H]HRP (37 kBq/ml). Mannitol (final concentration 5 mM) was introduced on both sides of the intestinal layer and [^{14}C]mannitol (12.2 kBq/ml) on the apical side only. Lastly, ^{22}Na was added apically to obtain a specific radioactivity of 14.8 kBq/ml. The three markers were applied simultaneously in the apical compartment and their transport was monitored by sampling 800 µl of the basal compartment at 30 minute intervals for 120 minutes, with replacement with fresh Ringer. Radioactivity was counted on 500 µl samples by liquid scintillation photometry, using three appropriate channels and triple label correction (^{3}H, ^{14}C, and ^{22}Na). The rate of intact HRP transport was determined by an enzymic assay on 200 µl aliquots, as described by Maehly and Chance.[16] [^{3}H]HRP equivalent fluxes were calculated using ^{3}H counts, and constituted the total amount of HRP transported in either intact or degraded form. Degraded HRP fluxes were then calculated as [^{3}H]HRP equivalent fluxes minus intact HRP fluxes. HRP fluxes were expressed in ng/h per cm^2, and mannitol and Na fluxes were calculated according to their specific radioactivity and expressed in µmol/h per cm^2 and µEq/h per cm^2 respectively. Flux values are given at steady state—that is, as the mean values during the second hour after addition of apical tracers.

NATURE OF ^{3}H LABELLED METABOLITES FORMED DURING [^{3}H]HRP TRANSEPITHELIAL TRANSPORT

In another set of experiments, HT29-19A cells were treated for 48 or 72 hours with IFNγ in the Transwell culture plates, or left untreated. Because transcytosis allows the transport of very small amounts of HRP, we used a larger exposed surface area in the Ussing chambers than that used in the transport experiments and a longer incubation time with [^{3}H]HRP to obtain improved sensitivity. Therefore intestinal monolayers were mounted in Ussing chambers for a period of 4.5 hours, with an exposed area of 3.5 cm^2, and exposed on each side to 12 ml oxygenated Ringer solution containing HRP at a final concentration of 0.4 mg/ml and 333 kBq of [^{3}H]HRP. Although a tendency to an increased conductance after 4.5 hours was observed, the electrical parameters remained within a range compatible with cell monolayer integrity. The basal compartments from six Ussing chambers containing control or IFNγ treated cells (6 × 12 ml) were then pooled and concentrated by passage through a solid phase extraction microcolumn (Sep-Pak cartridge C_{18}; Waters Chromatography, Millipore, Milford, MA, USA), which retained the proteins and peptides but not the amino acids. Briefly, the column was equilibrated with 10 ml acetonitrile and washed with water containing 10 ml 1% trifluoroacetic acid (TFA), and the 72 ml sample was acidified with TFA (final concentration 1%, v/v) and passed through the microcolumn at a flow rate of 10 ml/min. Next, the column was washed with 0.1% TFA, and salts and amino acids were recovered in the eluate. Lastly, the proteins and peptides retained were eluted from the column at a flow rate of 2.5 ml/min, with 4 ml 80% acetonitrile/20% water/0.1%TFA solution. The protein and peptide fraction was concentrated using a Speed-vac system at a low temperature, and adjusted to 200 µl of the appropriate buffer for reverse phase high performance liquid chromatography (RP-HPLC) or steric exclusion HPLC.

Because the ^{3}H equivalent HRP found in the basal compartment after transport may consist of amino acids, peptides, and modified or intact

HRP, we calculated the percentage of each tritiated metabolite derived from HRP after transepithelial transport. Considering that the total radioactivity of the basal compartment represents 100%, the percentage of amino acids was calculated by counting the radioactivity of the first microcolumn eluate and washing. The percentages of peptides and intact HRP eluted from the microcolumn were calculated after RP-HPLC separation as follows: each eluted peak was manually integrated and its relative percentage estimated using Borwin software (EG&G Instruments, Evry, France).

HPLC OF ^{3}H LABELLED METABOLITES

RP-HPLC

^{3}H labelled metabolites were first analysed by RP-HPLC, and separated according to their peptide hydrophobicity. We used a Vydac C_{18} column (218TP; 10 µm; 250 × 4.6 mm; 300 Å) and a Shimadzu HPLC system coupled to an LB506 Berthold in line counter for radioactivity detection. The acquisition, integration, and calculation of data were performed with Borwin software. Samples were eluted at 36°C at a flow rate of 1 ml/min. TFA was used as the ion pairing agent, and elution was performed with a gradient consisting of 100% buffer A to 100% buffer B over a 60 minute linear gradient. Buffer A consisted of 0.115% TFA in water, and buffer B of 0.1% TFA, 40% water, and 60% acetonitrile.

Steric exclusion HPLC

^{3}H labelled metabolites were also analysed by steric exclusion HPLC in order to determine their molecular mass. Accordingly, a Superdex Peptide PE 7.5 (300) column, adapted to the separation of peptides with molecular mass ranging from 100 to 7000 Da, was eluted at 30°C for 45 min with 0.1% TFA in 30% acetonitrile. A calibration curve was constructed using various molecular mass markers, including cytochrome *c* (12 400 Da, elution time (ET) 16.2 minutes), aprotinin (6500 Da, ET 19.1 minutes), vitamin B_{12} (1355 Da, ET 26.7 minutes), hexaglycine (360 Da, ET 32 minutes), triglycine (180 Da, ET 34 minutes), and glycine (75 Da, ET 36.6 minutes).

MEASUREMENT OF LYSOSOMAL ACID PROTEASES (CATHEPSINS) IN HT29-19A INTESTINAL CELLS

After three weeks of culture, cells from each filter (10^6 cells) were detached and homogenised in 70 µl ice cold 0.1% (v/v) Nonidet P40.

Thiol protease (cathepsins B and L) activity was measured using S2160 as substrate as described by Butterworth *et al.*[17] Fresh cell extracts (50 µl) were incubated for 30 minutes at 37°C with 100 µl 0.4 M sodium citrate, pH 5.2, 50 µl soybean trypsin inhibitor (12 mg/ml), 50 µl 24 mM dithiothreitol, and 400 µl 0.48 mM substrate S2160. The reaction was stopped with acetic acid (30%), and the mixture was centrifuged for 20 minutes at 4000 *g* and 4°C. The *p*-nitroanilide released was measured by spectrophotometry at 410 nm. Protein concentration was determined by the Lowry method.

Aspartyl protease activity (cathepsins D and E) was measured as described by Barrett and Heath.[18] Bovine haemoglobin was used as substrate, and aspartyl protease activity was the pepstatin A sensitive proteolytic activity. The reaction mixture contained 100 µl 2% (w/v) bovine haemoglobin, 50 µl 1M sodium formate, pH 3.5, 50 µl either 0.09 mM pepstatin A (Sigma) or distilled water, and 50 µl cell homogenate. Incubation lasted for 30 minutes at 45°C and was stopped with 500 µl 5% trichloroacetic acid. The supernatant was collected after centrifugation for 20 minutes at 4000 *g* and 4°C, and the tyrosine content was measured by the Lowry method.

HLA-DR EXPRESSION ON HT29-19A INTESTINAL CELL LINE

The surface expression of HLA-DR molecules was analysed using mAbs directed against class II DR epitopes. Briefly, after trypsinisation, 4×10^5 cells were incubated with 10 µl HLA-DR mAb (clone L243) for 20 minutes at 4°C in phosphate buffered saline. Negative controls with Leu4 mAb (anti-CD3) and without mAb were run concomitantly.

Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson). HLA-DR expression on the cell surface was measured by the shift in the fluorescence of fluorescein isothiocyanate labelled cells in relation to the shift in that of negative control cells.

STATISTICAL ANALYSIS

Results are expressed as means (SEM), with n = number of filters. Data were analysed using the SAS package (SAS institute, Cary, NC, USA).[19] Variance analysis was performed using the general linear model procedure, and least square means statement was used to compare means.

Results

TRANSPORT STUDIES ACROSS CONTROL AND IFNγ TREATED H29-19A INTESTINAL CELL MONOLAYERS

Ionic conductance and mannitol and Na fluxes

As shown in fig 1, in control conditions, ionic conductance (G), and mannitol and Na fluxes across HT29-19A intestinal monolayers were respectively 7.4 (0.3) mS/cm^2, 0.031 (0.001) µmol/h per cm^2, and 2.75 (0.10) µEq/h per cm^2. All these parameters increased in the presence of IFNγ placed on the basal side of the intestinal cells. When IFNγ was placed on the apical side, only the 72 hour period of treatment induced small but significant increases in ionic conductance, mannitol, and Na fluxes ($p<0.02$). These results confirm that IFNγ increases paracellular transport of small molecules when placed on the basal side of HT29-19A intestinal cells.

Total, intact, and degraded HRP fluxes

As fig 2 indicates, in untreated cells, the total flux of HRP—that is, ^{3}H equivalent HRP flux—was 3135 (219) ng/h per cm^2 and was composed of intact HRP flux (39.2 (6.2) ng/h per cm^2) and degraded HRP flux (3099 (215) ng/h per cm^2).

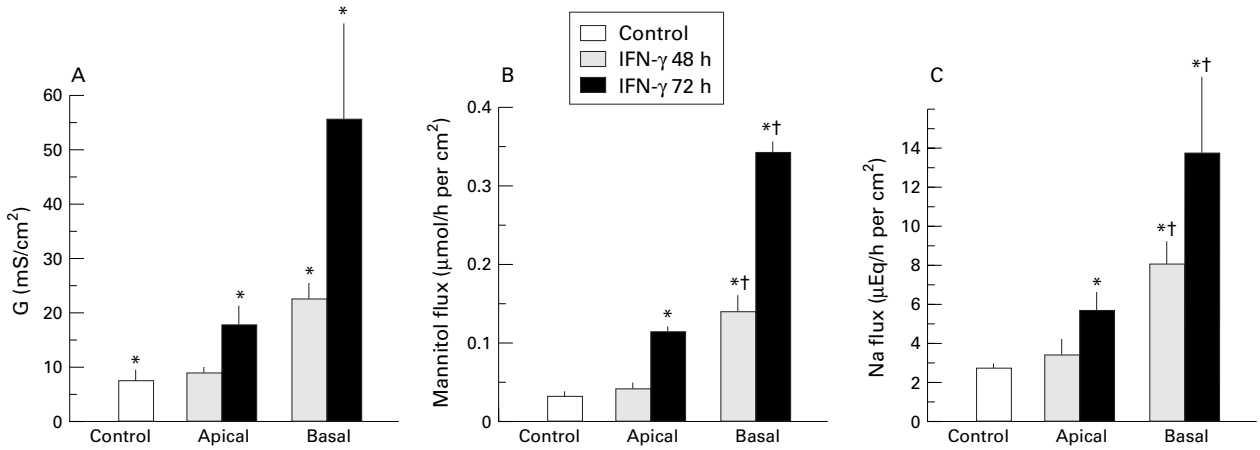

*Figure 1 Effect on paracellular transport of placing interferon γ (IFNγ) on the basal or apical side of HT29-19A intestinal cell monolayers for 48 or 72 hours. Paracellular permeability was measured as (A) ionic conductance (G), and (B) mannitol and (C) Na fluxes. *Significantly different from untreated cells (p≤0.02); †significantly different from time matched cells after IFNγ treatment on the apical side (p≤0.03). n = 8 to 20 filter grown intestinal monolayers.*

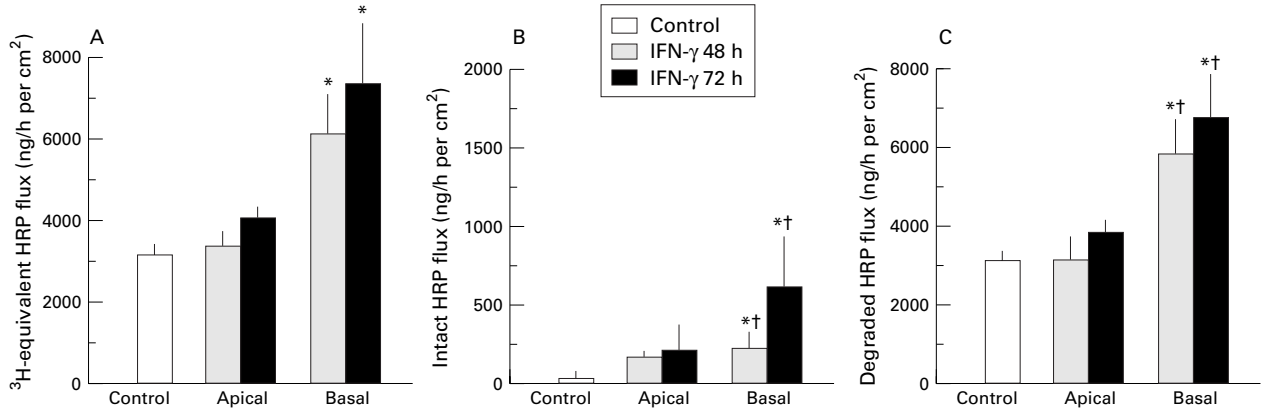

*Figure 2 Effect of placing interferon γ (IFNγ) on the basal or apical side of HT29-19A intestinal cell monolayers for 48 or 72 hours, on the transepithelial transport of (A) ^{3}H labelled horseradish peroxidase ([^{3}H]HRP) in (B) intact or (C) degraded form. *Significantly different from untreated cells (p≤0.008); †significantly different from time matched apical conditions (p≤0.01). n = 8 to 17 filter grown monolayers.*

Placing IFNγ in the basal compartment of the Transwell grown HT29-19A cells, for 48 or 72 hours, significantly enhanced ^{3}H equivalent HRP fluxes (to 7392 (1433) ng/h per cm^2). This enhancement was due to increases in both intact HRP absorption (618 (347) ng/h per cm^2) and degraded HRP (6774 (1125) ng/h per cm^2). The latter increase reflected an enhanced rate of transcellular transport with intracellular degradation since no degradation of the [^{3}H]HRP occurred in the apical compartment during the 4.5 hour experiment (see HPLC section), excluding the possibility of leakage of ^{3}H labelled metabolites through damaged tight junctions. When placed on the apical side of HT29-19A cells for 48 or 72 hours, IFNγ failed to induce any significant changes in HRP transport. These results show that IFNγ stimulates the transcytosis of HRP.

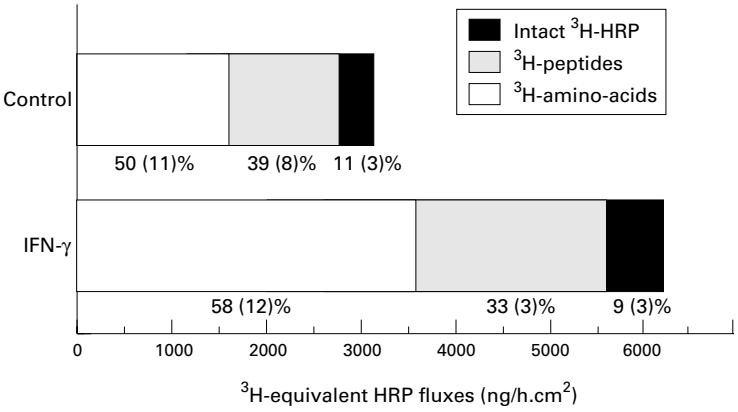

Figure 3 Processing of ^{3}H labelled horseradish peroxidase ([^{3}H]HRP) during transport. Incubation for 48 hours with interferon γ (IFNγ) increased ^{3}H equivalent HRP transport (total HRP) without significantly modifying the percentages of amino acids, peptides, and intact HRP. n = 5 to 6 separate experiments.

NATURE OF ^{3}H LABELLED METABOLITES FORMED DURING [^{3}H]HRP TRANSPORT

About 50% of the HRP transported was completely degraded into amino acids, 40% was processed into peptides, and 10% remained intact. Although IFNγ significantly increased ^{3}H equivalent HRP fluxes, the relative distribution between intact HRP, peptides, and amino acids in control and IFNγ treated cells remained unchanged (fig 3).

HPLC ANALYSIS OF ^{3}H LABELLED METABOLITES FORMED DURING [^{3}H]HRP TRANSPORT

RP-HPLC

Figure 4 shows the results of RP-HPLC, the most commonly used technique for the analysis of proteins and peptides, using TFA as the ionic pairing agent. Figure 4(A) represents the

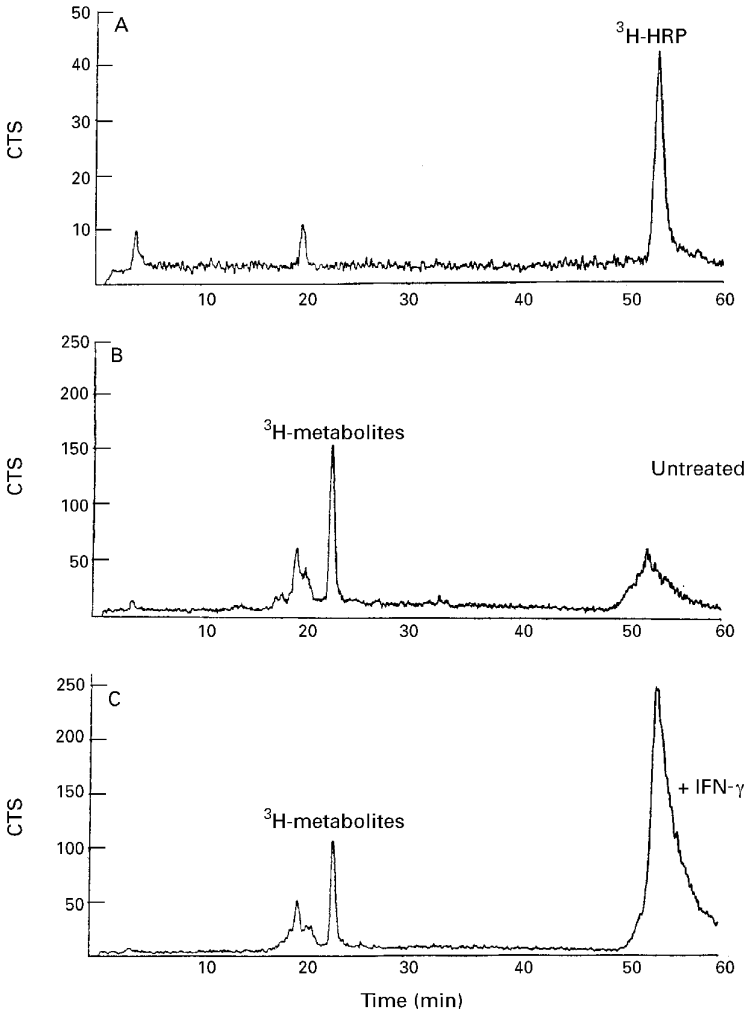

Figure 4 ^{3}H labelled metabolites formed after transepithelial transport of ^{3}H labelled horseradish peroxidase ([^{3}H]HRP) across intestinal HT29-19A cells, as shown by reverse phase high performance liquid chromatography (RP-HPLC). [^{3}H]HRP before transport (A), and ^{3}H labelled metabolites and [^{3}H]HRP after transport in untreated cells (B) and in cells treated with interferon γ (IFNγ) for 48 hours (C). This elution profile is representative of two separate experiments.

radioactive profile of intact [^{3}H]HRP before transport across HT29-19A intestinal cells and shows that it is eluted at 57.8 minutes. After 4.5 hours in the apical compartment of the Ussing chamber, the HPLC profile was similar, indicating that [^{3}H]HRP remained intact (not shown).

After transport under control conditions (fig 4B), the ^{3}H labelled products formed a wide peak which was eluted at the same time as intact [^{3}H]HRP, and a group of ^{3}H labelled metabolites eluted between 19.7 and 23 minutes. Figure 4(C) shows the profile of the ^{3}H labelled products eluted after IFNγ treatment: the intact [^{3}H]HRP peak is sharper, and a group of ^{3}H labelled metabolites roughly comparable with those found under control conditions is also eluted.

Steric exclusion HPLC

The ^{3}H labelled metabolites found after transepithelial transport were probably peptides, but RP-HPLC did not indicate their molecular mass, and it is possible that they constituted [^{3}H]HRP with a modified tertiary structure, modifying the interaction with the hydrophobic phase. To evaluate the molecular mass of these metabolites, we used steric exclusion HPLC. Figure 5(A) shows the elution profile of intact [^{3}H]HRP before transport. Intact [^{3}H]HRP (molecular mass 40 kDa) was eluted with the void volume at 17.6 minutes. Figure 5(B) shows the protein after 4.5 hours of incubation in the apical compartment bathing HT29-19A cells, and indicates that [^{3}H]HRP remained intact during the whole experiment. Figure 5(C) shows the ^{3}H labelled metabolites after [^{3}H]HRP transport. A wide range of degradation products with a continuous molecular mass gradient was eluted, indicating that many peptides were formed during transcytosis. Two groups of ^{3}H labelled metabolites were detected, one large group eluted from 16.2 to 24.4 minutes—that is, with molecular mass ranging from 40 to 2.5 kDa—and another group which peaked at an elution time of 28 minutes, corresponding to a molecular mass of 1150 Da—that is, peptides of about eight amino acids. No ^{3}H labelled amino acids are shown to have been eluted in these chromatograms, because amino acids were eliminated by the initial purification on a Sep-Pak column. Figure 5(D) shows the elution profile obtained after cell stimulation with IFNγ. It was very similar to the profile for untreated cells. The complexity of the elution pattern did not allow us to characterise important differences between the two conditions.

LYSOSOMAL ACID PROTEASE (CATHEPSIN) ACTIVITY

As shown in table 1, lysosomal aspartyl and thiol protease activities were found in basal conditions in HT29-19A intestinal cells. After 4, 48 or 72 hours of treatment with 100 U/ml IFNγ, their activities were not modified.

EXPRESSION OF HLA-DR MOLECULES ON HT29-19A INTESTINAL CELLS

After 48 or 72 hours of treatment of HT29-19A intestinal cells with 100 U/ml rhIFNγ on their basal side, cell viability was greater than 90%, as assessed by trypan blue exclusion. As shown in fig 6, no HLA-DR molecules were expressed in control cells—that is, the fluorescence was not different from that measured in the negative control without mAb. After 48 hours of treatment with IFNγ, placed on the basal side of the cells after 21 days in culture, HLA-DR molecule expression was moderate compared with its expression in unstimulated cells, and after 72 hours of treatment, it had risen notably. In contrast, when IFNγ was applied on the apical side, no such expression was observed (data not shown).

Discussion

The present results indicate that the intestinal epithelium is able to generate peptides during transcytosis of food type proteins, and that IFNγ, a proinflammatory cytokine, allows not only paracellular leakage, but also enhances the antigenic load transmitted to the intestinal

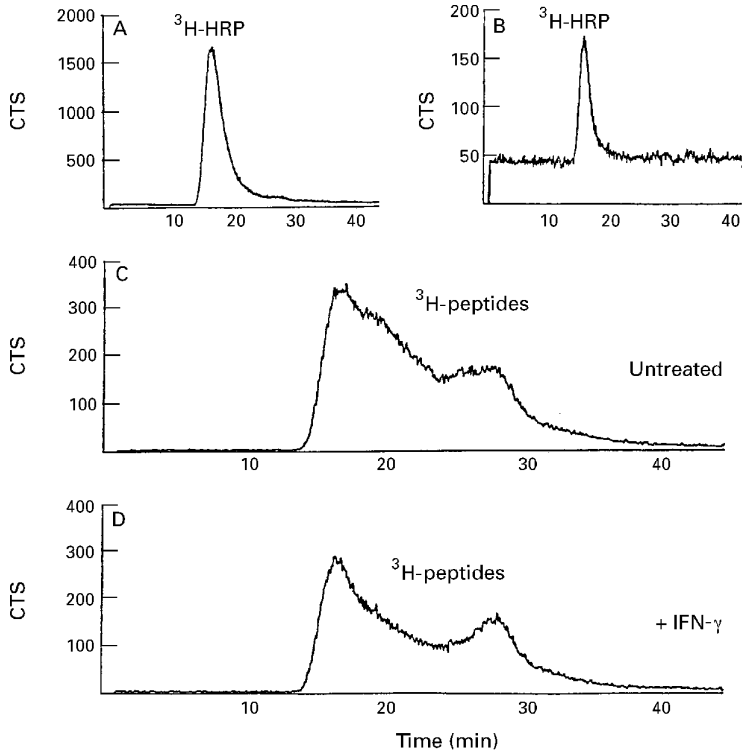

Figure 5 ^{3}H labelled peptides formed during transepithelial transport of ^{3}H labelled horseradish peroxidase ([^{3}H]HRP) across intestinal HT29-19A cells as shown by steric exclusion HPLC. [^{3}H]HRP before transport (A),[^{3}H]HRP after 4.5 hours in the apical compartment (B),[^{3}H]metabolites after transepithelial transport in the basal compartment, in untreated cells (C) and cells treated with interferon γ (IFNγ) (D). This chromatogram is representative of two separate experiments.

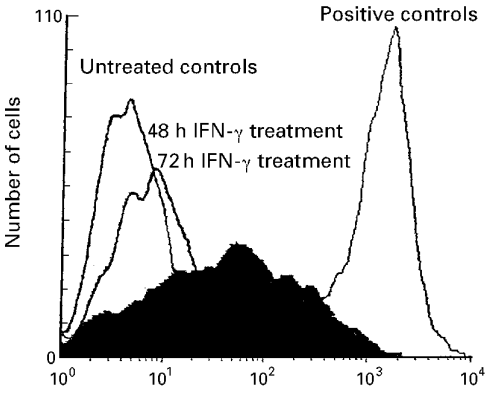

Figure 6 HLA-DR molecule expression on the surface of untreated intestinal HT29-19A cells, and cells treated on their basal side with 100 U/ml recombinant human interferon γ (IFNγ) for 48 or 72 hours. A monoclonal antibody recognising HLA-DR (clone L243) was used. Untreated cells did not express HLA-DR. After 48 hours of IFNγ treatment, HLA-DR expression was significant, and after 72 hours, it had greatly increased. A positive control was run with EBV-B cells.

mucosa by increasing the amount of peptides produced by the enterocyte.

Enterocytes are probably involved in the immune response to luminal antigens, because they can release, into the intestinal lamina propria, antigenic material in the form of peptides[20] capable of activating local immune cells, or of directly presenting antigens to intestinal T cells. It is not known whether peptides can be released from the MHC class II/peptide complex at the basal pole of the enterocytes, or whether other pathways allow their transepithelial processing and transport. However, the release of peptides, generated inside the enterocytes, into the intestinal lamina propria could have important consequences, because linear peptides of 5–20 amino acids can, at least in vitro, act as a substitute for intact antigens, in stimulating T cells.[21 22] In addition, the differential induction of immediate hypersensitivity and T cell proliferation due to changes in either peptide structure or MHC haplotype have been described in vivo.[23] Lastly, certain synthetic peptides have been reported to compete successfully with native peptides for binding to MHC class II molecules.[24] These observations highlight the need to study the role of the intestinal epithelium in the absorption and processing of luminal food antigens.

In the present study, we used the HT29-19A intestinal cell line because it has been shown to express MHC class II molecules[25] and to transport macromolecules.[26] We confirmed that in these cells, a small fraction of HRP is transported intact under basal conditions and showed that the remaining HRP—that is, most of it—is processed into amino acids and peptides during transcellular transport. On RP-HPLC, during which proteins and peptides are mainly separated according to their hydrophobicity, the molecules with a different elution time from that of intact HRP are likely to be peptides. However, they could also be conformationally modified HRP, since modifications in the tertiary structure of HRP might in turn modify its interactions with the separation phases. Using gel filtration HPLC, we found that intracellular HRP processing generated peptides with a wide range of molecular mass.

Table 1 Lysosomal aspartyl and thiol protease activities in untreated filter grown HT29–19A intestinal cells, and cells treated with 100 U/ml interferon γ (IFNγ) on the basal side for four to 72 hours

	Activity (nmol/min per mg protein)			
		+IFNγ		
Enzyme	*Untreated controls*	*4 hours*	*48 hours*	*72 hours*
Thiol protease	0.67 (0.06) (n=33)	0.64 (0.26) (n=5)	0.59 (0.10) (n=7)	0.86 (0.11) (n=6)
Aspartyl protease	12.97 (0.67) (n=21)	13.30 (1.70) (n=7)	14.35 (1.64) (n=7)	11.40 (2.21) (n=6)

Values are expressed as mean (SEM).
Thiol and aspartyl protease activities are respectively expressed in nmol p-nitroaniline/min per mg protein and nmol tyrosine/min per mg protein.
n, Number of filters.
No significant differences were found between treatment periods.

The expression of MHC class II molecules is normally a characteristic of conventional antigen presenting cells such as monocytes/macrophages and B lymphocytes, and is mandatory for peptide presentation to T cells. Various epithelial cells, including thyroid, bronchial, and intestinal cells,[10] express MHC class II molecules in pathological situations in which proinflammatory cytokines such as IFNγ and tumour necrosis factor α are secreted. However, distinct MHC class II molecules seem to be expressed by intestinal epithelial cells,[2] and are thought to play a role in regulating the mucosal immune response.[3 27 28] The low basal expression of MHC class II molecules in murine intestinal epithelial cells was associated with the absence of presentation of bovine serum albumin to specific T cells, whereas IFNγ induced MHC class II molecule expression enabled bovine serum albumin presentation.[29] Furthermore, IFNγ has been shown to regulate the processing and routing of MHC class II/peptides in a murine macrophage cell line.[30] In addition, colocalisation of these molecules and exogenous antigens in enterocytes has been reported, for both bovine serum albumin[31] and gliadin,[32] suggesting that a link exists between MHC class II molecules and the endocytic pathway in enterocytes, with a possible role in the handling of gut antigens.

In IFNγ treated HT29-19A intestinal cells, the surface expression of MHC class II molecules (HLA-DR) was significant. This expression was only induced effectively when IFNγ was placed on the basal side of the intestinal cells, confirming the basal location of the IFNγ receptors.[33] In addition, IFNγ enhanced HRP transport along both the paracellular and transcellular pathways. Again, basal but not apical IFNγ was responsible for these enhancements. It was previously reported that IFNγ disturbs the paracellular permeability of epithelia.[25 33] In vitro, the intestinal cell line T84 exhibited decreased electrical resistance, which was associated with an increase in mannitol fluxes, suggesting a leakage in paracellular tight junctions.[34] In the present study, we confirmed the ability of IFNγ to disrupt intestinal epithelial integrity. We also showed that IFNγ increases HRP transport in the intact form, and increases fluxes of degraded HRP, an index of transcellular transport. This increase in the transcytosis rate did not greatly change the relative proportions of the amino acids, peptides, and intact HRP transported. Although we did not determine the pathway involved in intact HRP transport in IFNγ treated cells, we suspect that it may involve the paracellular route. However, the increase in intact HRP fluxes remained small compared with the total quantity transported, indicating that the macromolecular leakage at the paracellular level was small compared with the total amount transported (~ 500 *v* ~7000 ng/h per cm^2).

Exogenous antigen processing is thought to occur mainly in the endosomal and lysosomal compartments, where acidic proteases—that is, cathepsins—are active. Cathepsins B and D have been shown to play a major role in the in vitro processing of antigens,[35 36] and IFNγ may regulate such processing.[37 38] However, in the present study, IFNγ had no effect on the activity of lysosomal acid proteases. The present work shows that enterocytic processing of [^{3}H]HRP leads to the formation of several populations of ^{3}H labelled peptides.This complex peptide profile did not allow us to assess the changes and differences that may have occurred in the ^{3}H labelled peptide profiles of control and IFNγ treated cells. Some of the peptides formed during transepithelial transport had a range of molecular mass compatible with binding to MHC class II molecules or to other restriction molecules, such as CD1d,[39] suspected to be involved in antigen presentation by enterocytes. The peptides linked to these molecules are linear peptides with a molecular mass lower than 2000 Da. The mechanism involved in the protection of such peptides from complete degradation within the enterocyte could be related to their linkage to some intracellular restriction molecules. In our study the generation of peptides with a molecular mass of about 1150 Da could be due to such protection, although the restriction molecule involved is not known, and is probably not related to MHC class II molecules, because even though MHC class II molecules—that is, HLA-DR—were exclusively expressed in IFNγ treated cells and not in untreated cells, almost the same peptide population was eluted in the relevant range of molecular mass by steric exclusion HPLC.

It is noteworthy that tritiation of HRP by reductive methylation of lysines[7] was used in the present study to enhance the sensitivity of the analysis and avoid the large background of unrelated peptides and proteins normally secreted by the enterocytes. Labelling of HRP by tritiation allowed us to detect some of the peptides formed during transport but perhaps not all, because only peptides containing a labelled lysine were detectable. Accordingly, it is not impossible that the absence of differences between the peptide profiles obtained in untreated and IFNγ stimulated cells may be due to the generation of unlabelled blind peptides. In conclusion, using an in vitro intestinal epithelial model, we suggest that the intestinal epithelium is able to generate peptides during transcytosis of intact proteins and that IFNγ, a proinflammatory cytokine, allows not only paracellular leakage, but also enhances the peptidic load transmitted to the intestinal mucosa. Analysis of the complexes formed between restriction molecules and ^{3}H labelled peptides inside the enterocytes may be another way of gaining further insight into the regulation of antigen transport and processing.

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