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The interaction of infant formula with macrophages: effect on phagocytic activity, relationship to expression of class II MHC antigen and survival of orally administered macrophages in the neonatal gut

A. HUGHES,* J. H. BROCK, D. M. V. PARROTT & F. COCKBURN† *University Department of Bacteriology and Immunology, Western Infirmary and †University Department of Child Health, Royal Hospital for Sick Children, Glasgow*

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SUMMARY

The effect of infant formula on human peritoneal and breast milk macrophages has been investigated. The ability of peritoneal macrophages to subsequently ingest and degrade immune complexes was slightly impaired, but breast milk cells were not affected. However, the cells were found to have bound antigenically intact casein and β-lactoglobulin, although little, if any, α-lactalbumin was bound. Furthermore, a positive correlation was found between binding of these proteins and expression of HLA-DR antigen. Labelled macrophages fed to newborn mice survived for at least 4 hr in the gastrointestinal tract and, in some cases, localized in the mucosal tissue. In one case a labelled cell was found in the spleen. These findings indicate that breast milk macrophages may be able to perform immunological functions in the gut, and suggest that binding of cows' milk proteins by macrophages may constitute a first step in the sensitization of the neonate to cows' milk proteins. Human milk macrophages may also play a protective role by acting as antigen-presenting cells in the local immune response of the gut.

INTRODUCTION

Greater awareness of the protective role of breast milk against neonatal infection has led to an increase in the number of mothers choosing to breast-feed their infants (Martinez & Nalezienski, 1980), but until recently little light has been shed upon the contribution made by immuno-competent cells in the milk. Pitt, Barlow & Heird (1977) suggested that milk macrophages may play a role in preventing necrotizing enterocolitis in the neonate, and showed that infant formula reduced the ability of rat milk leucocytes to kill bacteria. Recent research in our laboratory (Hughes *et al.*, 1985), aimed at investigating further the effect of milk on the phagocytic activity of macrophages, has shown that formula milk inhibits phagocytosis and degradation of immune complexes by resident mouse peritoneal macrophages, and a similar but less pronounced effect was observed on macrophages elicited with thioglycollate broth. Breast milk had little or no effect. Since immunofluorescence studies have shown that bovine casein and β-lactoglobulin bound to these cells it was considered that the inhibitory effect of the formula may be mediated by intact cows' milk proteins surviving the sterilization procedure and interfering with cell function. This inhibitory effect of formula, if applicable to human milk cells and gut-associated macrophages, may have important implications for infants on a mixed-feeding regime since these babies may be more susceptible to infection and more likely to develop sensitivity to cows' milk proteins than the wholly breast-fed infant. However, comparable studies on human macrophages have not been performed.

Breast milk macrophages may also play a role in the development of the neonatal immune system. Macrophages expressing class II MHC antigen (HLA-DR or Ia antigen) can act as antigen-presenting cells (Yamashita & Shevach, 1977). and breast milk macrophages are known to contain a HLA-DR$^+$ population (Leyva-Cobián & Clemente, 1984). Thus, breast milk macrophages, and perhaps mucosal macrophages, may help to initiate immune responses to ingested antigens. However, such an event may not necessarily be beneficial, as it might lead to hypersensitivity reactions. This would be true particularly of infants fed formula containing antigenically intact cows' milk proteins. Since we have shown previously that mouse peritoneal macrophages exposed to infant formula bind antigenically intact casein and β-lactoglobulin, it would clearly be of interest to determine whether HLA-DR$^+$ human macrophages also bind these proteins when exposed to formula, as both these proteins have been implicated in cows' milk allergy (Devey *et al.*, 1976).

The ability of breast milk macrophages to participate in protective and immunological activities in the neonate presup-

* Present address: Dept. of Pediatric Gastroenterology and Nutrition, Harvard Medical School, Massachussetts General Hospital, Boston, MA 4153, U.S.A.

Correspondence: Dr J. H. Brock, Dept. of Bacteriology and Immunology, Western Infirmary, Glasgow G11 6N, U.K.

poses that these cells reach and remain in the small intestine in a functionally active state, and perhaps even traverse the gut mucosa to reach the primary lymphoid organs. While there is some evidence that lymphocytes or milk cells in general may be able to do so (Seelig & Billingham, 1981; Weiler, Hickler & Sprenger, 1983) there does not appear to be any comparable study which has examined specifically the fate of macrophages.

The objectives of this study, therefore, were three-fold. Firstly, we wished to determine whether the phagocytic activity of human macrophages was affected adversely by prior exposure to infant formula, as found previously with mouse peritoneal macrophages (Hughes *et al.*, 1985). Secondly, we have investigated whether macrophages so exposed bound cows' milk proteins, and whether this was associated with HLA-DR$^+$ cells. Finally, we have examined the fate of orally administered macrophages in the neonate.

The ideal choice of cells for these studies would have been breast milk and gut mucosal macrophages. While the former can be obtained reasonably easily, it is not feasible to obtain human mucosal macrophages in numbers sufficient for a study of this type. Therefore, we have used human peritoneal macrophages as an alternative, taking advantage of the fact that these can be obtained in good numbers from the spent dialysis fluid of patients undergoing continuous ambulatory peritoneal dialysis (CAPD). To examine the fate of orally administered macrophages, we have developed a model system in which macrophages labelled with fluorescent latex beads are administered orally to newborn mice.

MATERIALS AND METHODS

Macrophage cultures

Colostrum and milk samples (1–5 days post-partum) were expressed from healthy mothers into sterile plastic containers and processed within 2 hr of collection. After centrifugation at 200 ***g*** for 10 min, the supernatant and lipid layer were removed from the sedimented cells and thoroughly mixed to disperse fat globules. The cell fraction was washed twice in sterile phosphate-buffered saline (PBS) and resuspended in culture medium which consisted of RPMI-1640 medium (Flow Laboratories, Irvine, Renfrewshire) supplemented with 5% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Flow Laboratories).

Human peritoneal macrophages were obtained from the spent peritoneal dialysate fluid of patients undergoing continuous ambulatory peritoneal dialysis (CAPD). Approximately 2 litres of dialysate were centrifuged in 1-litre buckets at 900 ***g*** for 15 min. The cells were collected, washed in sterile PBS and resuspended in culture medium. Viability of milk and peritoneal macrophages, as determined by eosin exclusion, was always $>95\%$.

Milk or peritoneal cells ($5–10 \times 10^5$ per well) were used to establish monolayers in either 48-well tissue culture plates (Costar, Northumbria Biologicals Ltd, Cramlington, Northumberland) or on 16-mm diameter glass coverslips. After incubation for 2 hr, non-adherent cells were removed, leaving approximately $2–3 \times 10^5$ adherent cells. These contained $>90\%$ macrophages, as determined by non-specific esterase staining (Yam, Li & Crosby, 1971).

Phagocytosis and degradation of ^{125}I-labelled transferrin–anti-transferrin immune complexes

The effect of breast milk and liquid infant formula on the ability of the macrophages to ingest and degrade labelled immune complexes was studied using the method described previously (Hughes *et al.*, 1985). The liquid infant formula used was Ostermilk Complete Formula (Farley Health Products, Plymouth, Devon) which has a casein:whey ratio of 77:23, similar to that of cows' milk (Farley Health Products, 1982) Briefly, adherent cells in 48-well tissue culture plates were exposed for 1 hr to milk, formula or, as a control, Hanks' buffered salt solution (HBSS), all diluted 50% (v/v) in culture medium. The cells were washed twice in HBSS and reincubated with fresh medium containing ^{125}I-labelled transferrin–anti-transferrin immune complexes (10 μg transferrin/well) for a further 2 hr. Uningested complexes were then removed and the cells reincubated in fresh medium overnight (18 hr). Non-adherent and dead cells were removed from the supernatants by centrifugation and an equal volume of 20% (w/v) trichloroacetic acid (TCA) added to the supernatant to precipitate any undegraded complexes.

The ^{125}I-activity of the TCA-soluble and insoluble fractions, the detached cells and the uningested complexes was measured using an LKB Compugamma gammacounter (LKB, Croydon, Surrey). The ^{125}I-activity within the adherent cells was determined following lysis in 2% (w/v) sodium dodecylsulphate. The number of replicates varied from experiment to experiment since it was dependent on the number of cells harvested from individual samples. Only samples that enabled a minimum of three replicates to be set up were used. Ingestion and digestion of immune complexes was calculated as follows:

$$\% \text{ ingestion} = \frac{T-U}{T} \times 100,$$

$$\% \text{ digestion} = \frac{S}{T-U} \times 100,$$

where T = total radioactivity added, U = radioactivity in uningested complexes and S = TCA-soluble radioactivity in the culture supernatant.

Immunofluorescence

Adherent macrophage cultures on glass coverslips were exposed to formula for 1 hr, washed twice, incubated with fresh medium for a further 30 min, and fixed in 2·5% formalin for 10 min. The macrophages were then washed in PBS for 5 min and incubated for 30 min at 20° in a moist chamber with rabbit antiserum to bovine casein, β-lactoglobulin or α-lactalbumin. All the sera used were prepared in the Department of Bacteriology and Immunology, Western Infirmary, except for the anti-α-lactalbumin, which was kindly provided by Dr A. Piñeiro, Department of Biochemistry, University of Zaragoza, Spain. The cells were then washed twice in PBS, incubated with fluorescent sheep anti-rabbit IgG and washed again. They were then stained for a further 30 min with a phycoerythrin-conjugated anti-human HLA-DR monoclonal antibody (Becton-Dickinson, Twickenham, Middlesex) and mounted in Tris-glycerol buffer (Thompson, 1977). A minimum of 100 cells were counted, scored as positive or negative for binding of milk protein, and then as positive or negative for expression of HLA-DR antigen. The number of cells in each category (HLA-DR$^+$, milk protein$^+$; HLA-DR$^-$, milk protein$^+$; HLA-DR$^-$, milk protein$^-$; HLA-DR$^+$, milk protein$^-$) was expressed as a percentage of the total

number of cells counted. Normal rabbit serum was substituted for antiserum to milk proteins in negative controls and gave < 1% positive staining. Staining for HLA-DR was standardized using HLA-DR$^-$ and $^+$ cell lines (K562 and BJAB, respectively). In order to ensure correct identification of cells staining positively with both fluorochromes, the cells were observed using excitation wavelengths of both 490 nm and 550 nm.

Labelling of macrophages with fluorescent latex beads

Macrophages were obtained by peritoneal lavage of adult CF/NIH mice and labelled by incubating 1 ml of cell suspension (10^7 cells) with 0·25 ml of a 1:200 dilution of a stock suspension of fluorescein-labelled latex beads (Polysciences Ltd, Northampton) for 45 min, with shaking. The cells were then sedimented by centrifugation at 170 *g* for 3 min, washed three times in PBS and resuspended in culture medium at 5×10^7 cells per ml. Microscopic examination revealed that about 85% of the cells contained at least six fluorescent beads, a figure which corresponded well with the proportion of the peritoneal cells identified as macrophages. Furthermore, all cells labelled with beads showed macrophage-like morphology when examined under phase contrast. The appearance of these cells labelled with multiple beads was quite distinct from the widely dispersed beads seen in tissues of neonatal mice dosed with free beads. The suspension contained no extracellular fluorescent beads.

Oral administration of macrophages to neonatal mice

Newborn (19–43 hr) CF/NIH mouse pups were fed $1–5 \times 10^6$ labelled cells in a 50-μl volume from a syringe fitted with a 25 gauge needle, to which was attached 1·5 cm of 00 gauge nylon i.v. tubing (Portex Ltd, Hythe, Hampshire). In order to prevent abrasion the end of the tube was smoothed by gentle heating and examined under a microscope before insertion into the oral cavity. Control animals were fed a similar volume of the diluted fluorescent bead suspension. After feeding the pups were returned to their mother.

Examination of stomach and intestinal contents

At appropriate time intervals pups were killed by decapitation and the stomach and small intestine removed. A smear of the stomach contents was made, after removal of any large lumps of milk curd. Sections (1–2 cm) were cut from the anterior end of the small intestine, cut longitudinally and the contents teased out into HBSS. Aggregates of milk curd were dispersed as far as possible by repeated passage through a Pasteur pipette, and the suspension (less any remaining large aggregates) transferred to a plastic bijou bottle. The suspension was centrifuged at 170 *g* for 3 min, washed twice in HBSS, resuspended in culture medium, and cytocentrifuge specimens prepared. Cells containing > six fluorescent beads were scored as positive.

Preparation of tissue specimens

Specimens of liver, spleen and the first 1–2 cm of the small intestine were snap-frozen in isopentane and prepared in OCT embedding compound (Raymond A. Lamb, London). Semi-serial sections (8 μm) were prepared, attached to glass slides and examined for the presence of labelled cells.

Statistical analysis

Stratified Sum of Ranks or the Chi-square test were used.

Table 1. Effect of human milk and infant formula on phagocytosis of immune complexes by human macrophages

	Percentage of immune complexes ingested		
Macrophages pre-incubated with:	50% HBSS (control)	50% human milk	50% formula
Breast milk macrophages	10·9 ± 4·5*	8·3 ± 6·6	11·1 ± 6·0
Peritoneal macrophages	10·4 ± 4·8	8·1 ± 3·5†	7·9 ± 3·7†

* Mean ± SD; $n \geq 3$.
† $P < 0·01$ compared with appropriate control.

Table 2. Effect of human milk and infant formula on degradation of immune complexes by human macrophages

	Percentage of ingested complexes degraded		
Macrophages pre-incubated with	50% HBSS (control)	50% human milk	50% formula
Breast milk macrophages	79·8 ± 27·1*	84·3 ± 30·8	77·7 ± 24·7
Peritoneal macrophages	67·8 ± 12·7	66·9 ± 15·7	62·2 ± 15·0†

* Mean ± SD; $n \geq 3$.
† $P < 0·01$ compared with appropriate control.

RESULTS

Effect of human milk and infant formula on phagocytosis and digestion of immune complexes by human macrophages

Breast milk macrophages. Breast milk macrophages showed a moderate ability to ingest immune complexes, and were unaffected by preincubation with liquid infant formula (Table 1). Breast milk supernatant caused a slight reduction in ingestion, but this was not statistically significant. Examination of the cultures microscopically before and after incubation with milk did not reveal any loss of cells.

Milk macrophages were capable of efficiently degrading internalized material (Table 2). Exposure of the cells to milk did not affect greatly degradation of the complexes.

Peritoneal macrophages. Phagocytic activity of human peritoneal macrophages was similar to that of breast milk macrophages (Table 1), and ingested complexes appeared to be efficiently degraded (Table 2). However, both colostrum supernatant and formula milk significantly inhibited phagocytosis. Digestion of the immune complexes was less susceptible to inhibition, although pre-exposure to formula caused a slight but statistically significant reduction.

Binding of cows' milk proteins and expression of HLA-DR antigen by macrophages

It was found that a proportion of human peritoneal macrophages exposed to formula stained positively for casein and β-

Table 3. Binding of casein or β-lactoglobulin and expression of HLA-DR antigen by human macrophages

Binding of:		% cells HLA-DR$^+$	% cells HLA-DR$^-$	Proportion HLA-DR$^+$ (%)	*P*
Peritoneal macrophages					
Casein	+	23·2±8·1*	5·8±2·9	80	<0·01†
	−	32·3±14·7	34·8±15·7	48	
β-lactoglobulin	+	12·9±4·7	4·4±3·6	75	<0·01
	−	40·8±20·2	42·0±21·6	49	
Breast milk macrophages					
Casein	+	16·3±9·8	3·2±3·4	84	<0·01
	−	59·3±28·0	21·3±16·8	74	
β-lactoglobulin	+	12·6±9·4	7·0±7·4	64	<0·05
	−	49·2±23·3	31·2±19·9	61	

* Mean ± SD; $n = 14$ for peritoneal macrophages and $n = 7$ for breast milk macrophages.

† The proportion of cells expressing HLA-DR was significantly greater in the population binding the relevant milk protein.

Table 4. Presence of orally administered macrophages in the stomach and duodenum of newborn mice

Time after feeding:	4 hr		21 hr	
Cells present in:	Stomach	Duodenum	Stomach	Duodenum
No. of animals:	35	29	22	21
Percentage of smears showing:				
No labelled cells	31	66	100	100
1–10 labelled cells	17	34	0	0
> 10 labelled cells	52	0	0	0

Cells were labelled prior to feeding with fluorescent latex beads. See text for further details.

lactoglobulin (28·6±8·0% and 18·0±3·7%, respectively; $n = 14$) but few, if any, of the cells bound α-lactalbumin (0·7±1·0%). All cells were negative when normal rabbit serum was substituted for antisera to milk proteins. The proportion of adherent cells expressing HLA-DR antigen varied between 8% and 82%, with a mean value of 55%. A positive correlation ($P < 0·01$) was found between binding of casein or β-lactoglobulin, and expression of HLA-DR antigen (Table 3).

Breast milk macrophages pre-incubated with liquid formula also stained positively for casein and β-lactoglobulin (19·5±12·1% and 19·6±15·0%, respectively; $n = 7$). These figures were not significantly different from the proportion found with peritoneal macrophages, though the results from individual samples were much more variable. However, a small proportion of cells from three of the seven donors also stained positively for α-lactalbumin (6·3±7·7%). The proportion of adherent breast milk cells expressing HLA-DR varied between 32% and 97%, with a mean value of 65%, which was slightly higher than that found with peritoneal cells. The correlation between expression of HLA-DR and binding of milk proteins by breast milk macrophages (Table 3) was again significant ($P < 0·01$ for casein; $P < 0·05$ for β-lactoglobulin). Binding of α-lactalbumin was too low to allow statistical testing.

Survival of orally administered macrophages in newborn mice

Labelled cells were regularly observed in the stomach smears of mice 4 hr after feeding, but none remained by 21 hr (Table 4). Some widely dispersed beads were also seen, indicating that some of the administered macrophages had lysed within the stomach. There were also large numbers of widely dispersed beads in the stomach smears of the controls. A similar pattern was observed with the duodenal washings, labelled cells being observed in several mice 4 hr after feeding but none at 21 hr.

Tissue specimens from four mice fed labelled macrophages were examined 4 hr later for the presence of labelled cells. In one of these, several labelled cells were observed within the mucosal tissue of the duodenum, and a single cell was observed in a spleen section. Tissues from the other three mice were negative. A single labelled cell was also seen in the duodenal mucosa of one out of two mice examined 21 hr after feeding the suspension. In no case were labelled cells observed in the liver. However, well-dispersed individual beads were observed in the livers of all but one of the mice examined, this pattern being similar to that found in the livers of control mice fed beads alone. This suggests that many of the administered macrophages were subsequently destroyed and released the ingested beads and that these were then absorbed and cleared by the liver. Dispersed beads were also seen in the mucosal tissue, indicating that at least some of this destruction of the administered cells occurred within the gastrointestinal tract.

DISCUSSION

In a previous study we reported that mouse peritoneal macrophages exposed to liquid infant formula showed a reduced ability to phagocytose and degrade radiolabelled immune complexes (Hughes *et al.*, 1985), and that this effect was associated with an interaction between intact bovine milk proteins present in the formula and the macrophages. The present study was undertaken to ascertain whether formula milk interacted similarly with human macrophages.

The ability of human peritoneal macrophages to ingest and degrade immune complexes was significantly reduced by pre-incubation with formula (Tables 1 and 2), as found previously with mouse peritoneal macrophages (Hughes *et al.*, 1985). However, an effect on ingestion was also observed when the cells were preincubated with cell-free human milk, and the effect of formula on degradation of the complexes, although statistically significant, was rather small. It should be noted that the peritoneal macrophages used in this study were all obtained from patients on CAPD who were suffering from renal failure, hence care must be taken in extrapolating these findings to normal peritoneal or mucosal macrophages. Peritoneal macrophages from CAPD patients appear to be relatively immature cells, resembling blood monocytes in a number of respects (Goldstein *et al.*, 1984; McGregor *et al.*, 1988)

The ability of breast milk macrophages to ingest and digest immune complexes was unaffected by preincubation with formula, their overall activity being similar to that of the peritoneal macrophages. Since these cells will already have been subjected to prolonged exposure to human milk proteins *in vivo* their phagocytic activity may have been reduced already to a point where subsequent exposure to cows' milk proteins has little effect.

Although infant formula caused at most only a modest decrease in the ability of human macrophages to handle immune complexes, a significant proportion of both breast milk and peritoneal macrophages were found to have bound casein and/or β-lactoglobulin. A small proportion of breast milk macrophages also bound α-lactalbumin, but this protein was not bound by peritoneal macrophages. This finding may be of relevance to the development of allergies to cows' milk proteins. In particular, it is of interest that casein and β-lactoglobulin have both been implicated in allergic responses, whereas α-lactalbumin is less likely to cause hypersensitivity (Devey *et al.*, 1976). Binding of casein and β-lactoglobulin by antigen-presenting macrophages may be an initial step in the development of hypersensitivity reactions. However, this would require that presentation of these antigens be carried out by HLA-DR-positive cells. The expression of this marker by human breast milk and peritoneal macrophages has not been widely studied, hence the presence of HLA-DR and its correlation with binding of milk proteins were investigated.

There was a wide variation between individual samples of peritoneal macrophages in the expression of HLA-DR. The mean value of 55% was somewhat lower than the value of 75% reported by Goldstein *et al.* (1984). Approximately 65% of breast milk macrophages expressed HLA-DR antigen. A slightly higher mean value of 80% was obtained by Leyva-Cobián & Clemente (1984) in the only comparable previous study; a lower figure of 23% reported by Mori & Hayward (1982) referred to total breast milk mononuclear cells rather than to macrophages. Variation in the sensitivity of the labelling techniques may be responsible for the other differences.

For both peritoneal and breast milk macrophages, it was found that cells binding casein or β-lactoglobulin showed a significantly increased tendency to express HLA-DR antigen. This indicates that the cells that are potentially capable of acting as antigen-presenting cells are also more likely to bind cows' milk proteins. Macrophages in the gut mucosa have been shown to express an Ia-like antigen (Golder & Doe, 1983), hence if a similar binding of milk proteins were to occur to these cells in the neonatal gut, it could constitute a first step in the development of an immune response and consequent hypersensitivity to these proteins.

Evidence that these *in vitro* effects may occur *in vivo* was provided by experiments in which labelled macrophages were fed to neonatal mice. In these experiments peritoneal cells were used rather than mouse milk cells because the latter are not well characterized and are difficult to obtain in large numbers. Moreover, it was desired to study the fate of macrophages rather than of other cell types, and the peritoneum provides a convenient source of a fairly pure population of these cells. The method of labelling consisted of ingestion of fluorescent latex beads, which not only served to identify the orally administered cells in smears and tissue sections, but also relies upon phagocytosis for labelling, thus selecting primarily for macrophages.

Orally administered macrophages were found regularly in stomach and duodenal contents 4 hr after feeding, but none remained at 21 hr. Evidence was also obtained that the orally administered cells could localize in the mucosal tissue, and the finding of a single intact labelled cell in the spleen of one mouse suggests that the occasional cell may even cross the gut wall and localize in peripheral lymphoid organs. Given that only a single cell was identified in peripheral organs, the possibility of an artifact cannot be ignored, but evidence for transfer of lymphocytes or milk cells in general has been reported by others (Seelig & Billingham, 1981; Sheldrake & Husband, 1985; Weiler *et al.*, 1983). There appears to be no previous report of the localization of orally administered macrophages in mucosal tissue. The possibility that the labelled cells were in fact of neonatal origin but had acquired beads released from the administered cells can be discounted, as the morphology of the orally administered labelled cells was very different from the pattern of dispersed beads seen in tissues and smears of controls fed a suspension of beads alone. However, dispersed beads were also observed in smaller numbers in the tissues of mice fed labelled cells, which suggests that not all cells survived passage through the gastrointestinal tract.

Since these experiments were not quantitative, the proportion of cells surviving in the gut or localizing in tissues cannot be ascertained. However, the fact that orally administered cells could be detected in an apparently healthy state in the duodenal mucosa lends support to the idea that breast milk cells may be able to act as antigen-presenting cells in the neonate, especially as this function may require only a small number of cells (Nunez, Ball & Stastny, 1983).

In conclusion, these results demonstrate that there is an interaction between infant formula and human macrophages, and the correlation between binding of cows' milk proteins and expression of HLA-DR suggests that this interaction may be involved in the development of harmful immune responses to cows' milk proteins. The high proportion of HLA-DR-positive macrophages in breast milk suggests that these cells could play a supportive role as antigen-presenting cells in the local immune response of the gut. This suggestion is strengthened by the demonstration that orally administered macrophages can survive in the gut of the newborn mouse and localize in mucosal tissues. Comparable studies on human mucosal macrophages, if these could be obtained in sufficient number, and investigation of their ability to act as antigen-presenting cells, may help to shed more light on their function *in vivo*. These results have also provided some preliminary evidence that orally administered macrophages may, like lymphocytes, cross the mucosal barrier and reach peripheral lymphoid organs. This possibility deserves further study.

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