Inability of normal human intestinal macrophages to form multinucleated giant cells in response to cytokines

S Fais, F Pallone

Abstract

Multinucleated giant cells are an important feature of the granulomatous reaction in Crohn's disease (CD) but their cellular origin is poorly understood. The aim of this study was to discover if intestinal macrophages are capable of generating multinucleated giant cells in vitro in response to cytokine stimulation. Human intestinal macrophages were isolated from the intestinal mucosa of CD and uninfamed surgical specimens. Isolated macrophages were cultured in chamber slides with and without exposure to a panel of cytokines and cell activators. Cell fusion, multinucleated giant cells formation, and the expression of adhesion molecules were assessed at various time intervals. In contrast with the autologous peripheral monocytes cell fusion was very poor in cultures of control intestinal macrophages and virtually no multinucleated giant cells were seen. Control intestinal macrophages seemed to poorly express the adhesion molecules required for cell to cell adhesion and fusion, namely ICAM-1 and LFA-1. None of these functions was affected by the exposure to cytokines, including interferon γ . In cultures of macrophages isolated from CD tissues multinucleated giant cell formation spontaneously occurred as early as three days and was not enhanced by the addition of cytokines. CD macrophages seemed to highly express both ICAM-1 and LFA-1. These data show that human intestinal macrophages are unable to form multinucleated giant cells in response to stimuli and support the concept that they are downregulated in a number of functions. The data also suggest that macrophages participating in the granulomatous reaction in CD are recruited from the circulation.

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Keywords: intestine, Crohn's disease, macrophages, cytokines, multinucleated giant cells, ICAM-1.

Multinucleated giant cells are a prominent feature of the granulomatous reaction in Crohn's disease (CD).^{1,2} The mechanisms and cellular requirements of multinucleated giant cell generation in CD tissues are not known. Recent evidence shows that 'in vitro' generation of multinucleated giant cells occur as a result of fusion of mature monocytes,^{3,4} and

that the addition of various cytokines,^{4–9} specific antibodies,¹⁰ and mitogens¹¹ modulate the ability of monocytes to fuse and form multinucleated giant cells. All human studies have been performed using peripheral monocytes, while the capability of human intestinal macrophages to form multinucleated giant cells is poorly understood. Interferon γ (IFN γ) has been shown to be more potent than other cytokines in inducing the fusion of peripheral blood monocytes and the subsequent multinucleated giant cell generation by promoting sequential changes in cell morphology and in the cellular expression of adhesion molecules.^{4–6} Time course experiments with IFN γ have shown that monocyte clustering and cell to cell adhesion are important sequential steps and that the polarisation of ICAM-1 on a cell uropode is essential for monocyte adhesion and fusion.⁴ As IFN γ expression and release are increased in the inflamed gut tissues in CD, it seems conceivable that IFN γ plays a part in the generation of multinucleated giant cells in CD.^{12–14} The aim of this study was to evaluate the ability of macrophages isolated from the intestinal mucosa of uninfamed and CD tissues to undergo cell fusion and form multinucleated giant cells in response to IFN γ and other cytokines.

Methods

Cell isolation and culture

The source of intestinal lamina propria mononuclear cells were the affected areas of 10 resected specimens of CD patients (six distal ileum and four colon) and the unaffected areas of surgical specimens of 16 colonic cancer and four diverticular disease patients. Lamina propria mononuclear cells were isolated using the DTT-EDTA-collagenase sequence as previously described.¹⁵ Both lamina propria mononuclear cells and autologous peripheral blood mononuclear cells were resuspended in RPMI 1640 medium (Flow Lab, UK) supplemented with 10% heat inactivated immunoglobulin deprived human pooled serum, L-glutamine, and antibiotics. Cells were then placed in tissue culture chamber slides (Lab-Tek, USA) (1.0×10^6 cells/chamber), coated with 0.2 ml prewarmed human pooled serum. Chambers were incubated for 90 minutes at 37°C in 5% CO₂ humid atmosphere and non-adherent cells washed off with warm RPMI 1640. The resulting adherent cells consisted of >90% macrophages and <3% T cells by Giemsa stain and by immunocytochemistry with anti-CD3

Laboratorio di
Virologia, Istituto
Superiore di Sanità,
Rome
S Fais

Dipartimento di
Medicina
Sperimentale,
Università di R
Calabria, Catanzaro,
Italy
F Pallone

Correspondence to:
Professor F Pallone,
Dipartimento di Medicina
Sperimentale, Policlinico
Universitario, Via T
Campanella, 88100
Catanzaro, Italy.

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Cytokine driven clustering, cell fusion, and multinucleated giant cell generation in 10 day cultures of intestinal and peripheral slide adherent monocytes/macrophages. Clustering is expressed as the number of clusters per unit area (see methods), cell fusion as fusion index (%) (see methods) and multinucleated giant cells as the number of multinucleated giant cells per unit area (see methods). Data are mean (SEM)

	Clusters		Fusion		Multinucleated giant cells	
	Intestinal	Peripheral	Intestinal	Peripheral	Intestinal	Peripheral
IFN γ	0	28(3)	0	78(4)	0	14(4)
IFN γ	0	0	0	0	0	0
IL1 α	0	23(10)	0	4(2)	0	6(2)
IL1 β	0	0	0	0	0	0
IL4	0	1(1)	0	2(1)	0	1(1)
IL6	0	24(9)	0	40(5)	0	12(3)
PMA	0	10(2)	0	8(3)	0	5(2)

PMA=phorbol-myristate-acetate.

and anti-CD68 MoAb. Average cell yield was 1.6×10^5 /chamber in both peripheral and intestinal cultures.

Multinucleated giant cells formation

Slide adherent macrophages were cultured in the presence of various concentrations of the following: human recombinant IFN γ (Boehringer Biochemia, Germany) (3, 10, 30, 100, 300, 1000 IU/ml); IFN γ (Bender MedSystem, Austria) (5, 10 IU/ml); IL1 α (50, 200 pg/ml), IL1 β (5, 20 pg/ml), IL4 (10, 100 ng/ml); and IL6 (2, 30 ng/ml) (Genzyme, USA). Phorbol-myristate-acetate (1 μ g/ml) stimulation was also provided to compare mitogenic with cytokine stimulation. Aliquots of cells were preincubated with purified anti-IFN γ monoclonal antibody (Hoffman-La Roche, Switzerland) at fourfold excess¹³ or with anti-IL1, IL4, IL6 MAbs (Genzyme Co, USA) as appropriate. Cells were checked daily for clustering and fusion using an inverted microscope (Leitz Willovert). At 3, 5, 7, and 10 days of culture, the medium was removed from the slide chambers and adherent cells were fixed and stained with Giemsa. Macrophage fusion rate was determined by counting the number of nuclei within multinucleated giant cells (>3 nuclei/cell) in a given area and the total number of nuclei in the same area.⁴ The fusion index was calculated according to the following formula: fusion index (%)=(number of nuclei within multinucleated giant cells)/(total number of nuclei counted) $\times$ 100. Using a 25 $\times$ objective and a 10 $\times$ eyepiece (Leitz Orthoplan Microscope) 500–800 nuclei from selected representative fields were counted for each experiment. The number of clusters were counted in each chamber area, using a grid mounted in the 10 $\times$ eyepiece with a low magnification (10 $\times$) objective thus permitting the observation of whole chamber slide. Counts were performed moving from the left upper square of the grid down to the lower one enumerating only clusters within squares. The final number was the sum of the clusters in each square.

Immunocytochemistry

Immunophenotyping of fixed adherent macrophages was performed using the APAAP method.^{4 16} The following MAbs were used as first layer antibody: anti- CD54 (ICAM-1,

Immunotech International France, clone 84H10); anti-CD11a (LFA1, Dako Denmark, clone MHM24); anti-CD68 (monocytes/macrophages, Dako Denmark, clone EBM11). A rabbit-antimouse MAb (Dako, Denmark) was used as a second layer followed by the APAAP complex (Dako, Denmark). New Fuxin (Merk, Darmstadt, Germany) was used as chromogen. Negative controls were cells treated with the second layer antibody only, with the APAAP complex alone, and with irrelevant isotype matched antibodies. Mayer's haematoxylin was used as counterstain.

Results

Cytokine driven multinucleated giant cell generation

Intestinal macrophages did not seem to cluster after exposure to any of the cytokines tested, nor with phorbol-myristate-acetate (Table). There was virtually no fusion in the slide chamber adherent intestinal macrophages and no multinucleated giant cells were seen (Table). In contrast, both IFN γ and IL6 seemed to dramatically enhance the generation of multinucleated giant cells in culture of the autologous peripheral monocytes (Table). The effect of IFN γ and IL6 as assessed by fusion index, number of clusters and multinucleated giant cells, was dose and time dependent. With both cytokines, clustering of macrophages, cell adhesion and fusion were important sequential steps. Anti-IFN γ Mab abrogated the effects of both IFN γ and IL6. The fusion index in the anti-IFN γ treated cultures did not differ to that of the unstimulated cultures (fusion index 0–2%) at any time and with any IFN γ and IL6 concentrations. Anti-IL6 Mab affected only IL6 driven multinucleated giant cell generation (not shown). In contrast, CD intestinal macrophages spontaneously formed multinucleated giant cells as early as three days after cultures with no cytokine addition. The fusion index ranged between 22% and 35% and the number of multinucleated giant cells from 11 to 28 per chamber at three days.

ICAM-1 and LFA-1 on adherent macrophages, fused macrophages, and multinucleated giant cells

Cells were examined by indirect immunocytochemistry after five days of culture with and without the addition of cytokines. Virtually all adherence purified macrophages were CD68 positive. ICAM-1 and LFA-1 were poorly expressed mean (SEM) 8 (3) % and 5(3) %, positive cells, respectively) by the CD68+ intestinal macrophages. The expression and cellular distribution of both ICAM-1 and LFA-1 on intestinal macrophages was not affected by the exposure to any of the cytokines tested in this study. After exposure to IFN γ the percentages of positive macrophages were 6 (1) % for ICAM-1 and 5 (4) % for LFA-1. In contrast, most peripheral monocytes strongly expressed both ICAM-1 (60 (4) %) and LFA-1 (81 (5) %). As previously described,⁴ in response to IFN γ and IL6, ICAM-1 was

upregulated and polarised on the cell membrane region where cell to cell contact eventually occurred. When these cytokines were added seven days after culture no ICAM-1 enhancing effect was seen. Immunohistochemistry of CD tissue sections showed that the proportion of intestinal macrophages expressing both ICAM-1 and LFA-1 was much higher 89 (6) % and 92 (7) %, respectively), and that ICAM-1 tended to be polarised on cell uropode.

Discussion

Results of this study show that normal human intestinal macrophages are not able to form multinucleated giant cells in response to various cytokines. Consistent with this finding intestinal macrophages poorly expressed ICAM-1 and its counter-receptor LFA-1 whose expression was not affected by the exposure to any of the cytokines tested. As the enhancement of ICAM-1 and LFA-1 and the subsequent polarisation of ICAM-1 are required for multinucleated giant cell generation 'in vitro', it is conceivable that the inability of intestinal macrophages to form multinucleated giant cells in vitro is related to the lack of adhesion molecules enhancement. This would be in agreement with various findings showing that intestinal macrophages are downregulated in their capability of exerting a number of functions. In fact, normal intestinal macrophages are not able to express IL2R,¹⁷ to develop respiratory burst activity,¹⁸ to produce IL1,¹⁹ to release IFN γ ,²⁰ and express IFN γ mRNA.¹² Of interest, these functions are spontaneously activated in macrophages isolated from CD tissues.¹²⁻¹⁷⁻²⁰ We have shown here that CD intestinal macrophages spontaneously form multinucleated giant cells and that in CD tissues the expression of both ICAM-1 and LFA-1 is enhanced.

In CD intestinal mucosa a spontaneous secretion of IFN γ and increased IFN γ mRNA has been shown¹²⁻¹⁴ and other putative 'in vitro' effects of IFN γ have been shown, such as HLA-DR expression on intestinal epithelium,²¹⁻²³ further suggesting that the inability of intestinal macrophages to 'in vitro' form multinucleated giant cells may well reflect an intrinsic cellular defect. The relevance of this finding to the granulomatous inflammatory reaction in Crohn's disease needs further investigation, as the responsiveness of macrophage/monocyte cells to IFN γ is important in host resistance to infection, as shown by recent experiment with *Leishmania* in murine system.²⁴

In conclusion, data of this study further support the view that normal human intestinal macrophages are cells downregulated in their capability to undergo fusion and multinucleated giant cell generation in response to IFN γ . This hypofunctional/hypo-responsive condition does not seem to be reversible, suggesting that tissue specific soluble factors may not be involved. Taken together, our data add indirect support to the concept that all the monocytes activated functions shown in CD

tissues, including multinucleated giant cells generated, may result from peripheral monocyte recruitment and activation into the site of inflammation.²⁵⁻²⁶

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