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Effect of human intestinal macrophages on immunoglobulin production by human intestinal mononuclear cells isolated from patients with inflammatory bowel disease

K. C. WU, Y. R. MAHIDA, J. D. PRIDDLE & D. P. JEWELL *Gastroenterology Unit, Radcliffe Infirmary, Oxford, England*

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SUMMARY

The effect of macrophages on spontaneous immunoglobulin production by isolated human intestinal mononuclear cells (MNC) is unknown. Depletion of macrophages by adherence to fibronectin or by panning with macrophage-specific monoclonal antibody 3C10 lead to a significant reduction in IgA, IgG and IgM production by intestinal MNC from both normal ($n=10$) and inflammatory bowel disease (IBD) ($n=13$) mucosa. The reduction in immunoglobulin produced by macrophage-depleted intestinal MNC was greater in IBD patients than in normal controls. There was a significant correlation ($r=0{\cdot}816$, $P<0{\cdot}001$) between the percentage of macrophages depleted by panning with 3C10 and the reduction in IgG produced by macrophage-depleted intestinal MNC. Addition of either fibronectin-adherent cells or the supernatant from these macrophage-enriched cells enhanced immunoglobulin production in a dose-dependent fashion. A greater increase in IgG production by macrophage-depleted cells was seen when cultured with supernatant from inflamed IBD mucosal cells, compared with that from normal mucosal cells. The soluble factor(s) responsible in the supernatant was acid and heat susceptible but was not affected by freezing and thawing. Addition of recombinant human interleukin-1β or human interferon-gamma to cell cultures did not influence immunoglobulin production. Thus, human intestinal macrophages enhance spontaneous immunoglobulin production by isolated intestinal MNC by secreting soluble factor(s) which remain to be fully characterized.

Keywords inflammatory bowel disease intestinal mononuclear cells

INTRODUCTION

Human intestinal macrophages are a group of cells heterogenous in morphology as well as histochemistry (Selby *et al.*, 1983). The function of these cells is not clear; it has been demonstrated that the intestinal macrophages are able to phagocytose (Kujawa *et al.*, 1977), to undergo respiratory burst (Mahida *et al.*, submitted for publication), to express interleukin-2 (IL-2) receptors (Mahida *et al.*, 1988b), and to present antigen (Mahida, Wu & Jewell, 1988a). Immunoregulatory properties of the cells have been suggested (Verspaget *et al.*, 1988).

Isolated intestinal mononuclear cells (MNC) spontaneously produce immunoglobulins *in vitro*, and the pattern of immunoglobulin production is altered in inflammatory bowel disease (IBD) (Bull & Bookman, 1977; MacDermott *et al.*, 1981; Wu *et al.*, 1989). Immunoglobulin production is likely to be regulated by cytokines produced by many kinds of immunocytes, including macrophages (Takemura & Werb, 1984; Ceuppens & Stevens, 1986; Murray *et al.*, 1987). In this study, the effect of intestinal macrophages on immunoglobulin production by isolated intestinal MNC was studied in patients with ulcerative colitis and Crohn's disease.

Correspondence: Dr D. P. Jewell, Gastroenterology Unit, Radcliffe Infirmary, Oxford OX2 6HE, England.

MATERIALS AND METHODS

Patients

Normal and inflamed intestinal mucosa was obtained from patients undergoing surgical resection either for carcinoma ($n=10$) or for severe IBD (seven with ulcerative colitis and six with Crohn's disease). The normal mucosa was taken from an area at least 5 cm from the tumour. All the patients with IBD, except one, were on corticosteroids at the time of the operation. All the specimens were examined histologically and the diagnosis confirmed.

Isolation and preparation of intestinal MNC

Intestinal MNC were isolated from normal and inflamed mucosa as described previously (Wu *et al.*, 1989). Briefly, mucosal strips were dissected from fresh operation resection

specimens and incubated with 1 mM dithiothreitol (DTT, Sigma, Poole, UK). Epithelial cells were removed by incubation of mucosa with 5 mM EDTA (BDH, Poole, UK) for 90 min with three washes in between. The mucosa was then minced and digested with collagenase (from *Clostridium histolyticum*; Boehringer, BCL, Lewes, UK) at a concentration of 1 mg/ml for 3h. The digestion was filtered and centrifuged on Ficoll–Paque (Pharmacia, Uppsala, Sweden). Intestinal MNC were collected from the interface of the centrifugation and washed and resuspended in culture medium (10% fetal calf serum (FCS) in RPMI, GIBCO, Paisley, UK).

In some experiments, MNC adherent and non-adherent to fibronectin-coated Petri dishes were obtained (Lin & Gordon, 1979; Bevilacqua *et al.*, 1981). A 0·1% solution of gelatin (Sigma) in distilled water was prepared by heating in a boiling water-bath for 15 min. Plastic Petri dishes (50 mm, Sterilin, Feltham, UK) were coated by adding 2 ml of cooled (to 37°C) gelatin solution and incubated at 37°C for 2 h. After aspiration of excess gelatin solution, the Petri dishes were dried for 2 h before incubating with 50% fresh normal human plasma (in RPMI) at 37°C for 1 h. The Petri dishes were then washed three times with RPMI before incubating with intestinal MNC for 1 h at 37°C. Fibronectin non-adherent cells were collected by gentle washing with warm (37°C) 5% FCS in RPMI. The removal of non-adherent cells was monitored by using an inverted microscope. The fibronectin-adherent cells were incubated at 37°C in 10% FCS/RPMI for about 12 h. They were detached by incubation at 4°C for 1 h followed by vigorous pipetting. Human monocytes were prepared in a similar way, from peripheral blood of healthy volunteers.

In some experiments, the intestinal MNC were depleted of macrophages by a panning technique (Wysocki & Sato, 1978) using a macrophage-specific monoclonal antibody 3C10 (IgG, gift from Dr R. Steinman, Rockefeller Institute, New York). Plastic Petri dishes were coated with goat anti-mouse IgG (Zymed, San Francisco, CA) at a concentration of 50 μg/ml in 0·05 M Tris buffer, pH 9·5, overnight at 4°C. Intestinal MNC (2×10^7) were incubated with antibody 3C10 at 4°C for 30 min and washed three times in 5% FCS/RPMI. The coated Petri dishes were washed with cold RPMI before adding the MNC. Following incubation at 4°C for 2 h, cells not bound to the Petri dishes were removed by gentle washing with cold (4°C) 5% FCS in RPMI.

Cytospin preparations for the isolated intestinal MNC and the other fractions of cells were made by a cytocentrifuge (Shandon, London, UK). They were air dried and fixed with acetone (BDH) for 5 min and stored at −20°C until used for staining. Subpopulations of MNC were studied by staining with the following mouse anti-human monoclonal antibodies: Y1/82A (monocyte and macrophage specific, kindly given by Dr D. Mason, John Radcliffe Hospital, Oxford); 3C10; T910 (*pan*-T, Dakopatts, High Wycombe, UK); T310 (anti-CD4, Dakopatts); DK25 (anti-CD8, Dakopatts) and T015 (*pan*-B, Dakopatts). Y1/82A and 3C10 were stained using the peroxidase technique (Gatter, Falini & Mason, 1982) and the rest using the alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique (Cordell *et al.*, 1984). The percentage of positive cells was determined by counting at least 200 MNC per population.

Immunoglobulin production in cell culture

Unfractionated intestinal MNC, fibronectin-adherent and non-adherent cells, and cells after panning with monoclonal antibody 3C10 were resuspended in RPMI supplemented with 10% FCS and antibiotics. The cells were then incubated for 12 days at a concentration of 1×10^6/ml at 37°C with 5% CO_2. The viability of the cells was >85% initially and >75% at day 12 of incubation, detected by 0·1% trypan blue exclusion. The concentration of IgG, IgA and IgM in the supernatants was measured by ELISA as previously described (Wu *et al.*, 1989).

In some experiments, various concentrations of fibronectin-adherent cells or the supernatants from them were cultured for 12 days with either fibronectin non-adherent cells or cells after panning with 3C10. Recombinant human interleukin-1β (IL-1β, Genzyme, Haverhill, UK), human interferon-gamma (IFN-γ, generous gift from Dr G. Scott, Wellcome Research Laboratories, Beckenham, UK) and the supernatant from cultured human monocytes were also used for incubation with fibronectin non-adherent cells or cells after panning with 3C10.

Characterization of supernatant from cultured fibronectin-adherent intestinal MNC

Supernatants from 12-day cultures of fibronectin-adherent cells were obtained by centrifugation at 11 500 ***g*** for 2 min followed by filtration through a 0·2-μm filter. The supernatants were freeze-dried and then stored at −20°C.

Freeze-dried supernatants were dissolved in a small volume of distilled water and trifluoroacetic acid was used to acidify the supernatants. Trifluoroacetic acid (50%, v/v) was added drop-by-drop into the stirring supernatant by a magnetic stirrer until the pH became 2·0. The acidified supernatant was left at room temperature for 30 min and neutralized to pH 7 by adding 1 M sodium hydroxide drop-by-drop while stirring. The acid-treated supernatant was then filtered and stored at −20°C till tested.

The freeze-dried supernatants were also treated with heat in a boiling-water bath for various time lengths from 1 to 30 min. The heat-treated supernatants were then filtered and stored at −20°C till tested.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or Wilcoxon signed rank, as indicated.

RESULTS

Effect of macrophage depletion on immunoglobulin production

The percentage of macrophages detected by staining with antibodies 3C10 and Y1/82A was significantly ($P < 0·01$) reduced in the fibronectin non-adherent cell population (8·7+1·3%, mean+s.e.m.) and in the cell population after panning with monoclonal antibody 3C10 (4·3+0·7%) compared with unfractionated intestinal MNC (15+1·6%). However, the percentage of macrophages in fibronectin-adherent cells (39+5%) was markedly enhanced ($P < 0·01$) in comparison with intestinal MC (Fig. 1). There was no significant difference in the percentage of T ($CD3^+$) cells, B ($CD22^+$) cells and the ratio of $CD4^+$ to $CD8^+$ cells between intestinal MNC and macrophage-depleted fibronectin non-adherent cells or cells after panning with 3C10. The percentage of T and B cells in macrophage-enriched fibronectin-adherent cells (16+4% and 4+0·7%) was significantly lower ($P < 0·01$) than in intestinal MNC (47+3·3% and 17+2·5%).

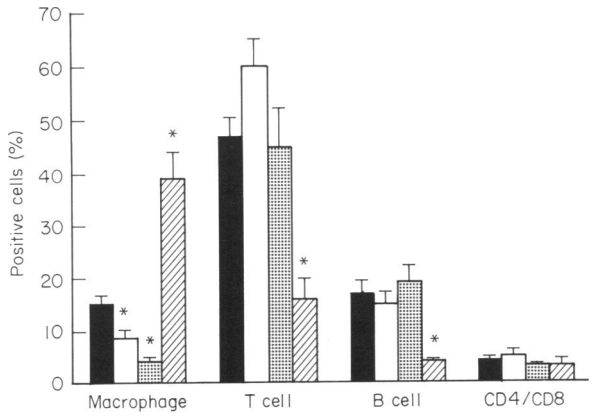

Fig. 1. Percentage of positive cells stained with monoclonal antibodies against macrophage, T cell ($CD3^+$, $CD4^+$, $CD8^+$) and B cell ($CD22^+$) in unfractionated intestinal mononuclear cells (MNC) (■, $n=13$); fibronectin non-adherent (NAC) (□, $n=12$); adherent cells (AC) (■, $n=8$); and cells panned of macrophages with antibody 3C10 (pan.) (▨, $n=7$).

* Significance $P<0.01$, macrophages in MNC *versus* NAC; macrophages in MNC *versus* pan.; macrophages in MNC *versus* AC; T cells in MNC *versus* AC; and B cells in MNC *versus* AC.

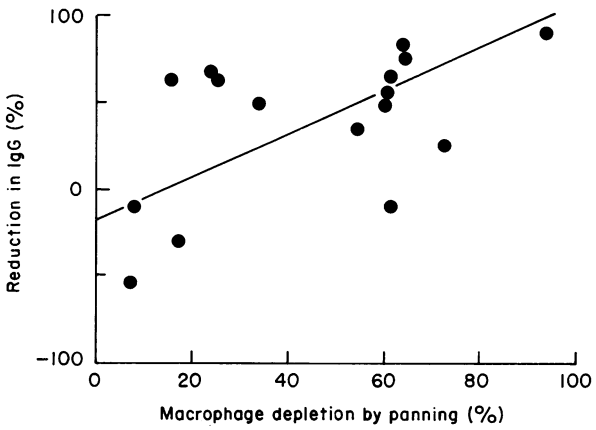

Fig. 2. Correlation of percentage macrophages depleted by panning with the percentage reduction in IgG produced by macrophage-depleted intestinal mononuclear cells.$n=17$; $P<0.001$, $r=0.816$.

Table 1. Immunoglobulin production (ng/ml) by intestinal mononuclear cells (MNC) and by fibronectin non-adherent cells (NAC) from control and IBD mucosa

	Controls ($n=6$)		IBD ($n=11$)	
	MNC	NAC	MNC	NAC
IgA	6286±1059	4454±1202*	1165±339	756±178†
IgG	410±55	225±59*	2634±880	1870±863†
IgM	1593±783	1162±724*	862±332	337±103*

IBD, inflammatory bowel disease.
* $P<0.05$, Wilcoxon signed rank test.
† $P<0.01$, Wilcoxon signed rank test.

Table 2. Immunoglobulin production (ng/ml) by intestinal mononuclear cells (MNC) and cells depleted of macrophages by panning with monoclonal antibody 3C10 from control and IBD mucosa

	Controls ($n=6$)		IBD ($n=13$)	
	MNC	Panning	MNC	Panning
IgA	5147±1228	3306±892*	2075±489	591±87 †
IgG	524±58	288±74*	1976±496	874±372†
IgM	586±118	242±43*	1336±347	493±199†

IBD, inflammatory bowel disease
* $P<0.02$, Wilcoxon signed rank test.
† $P<0.001$, Wilcoxon signed rank test.

Table 3. Immunoglobulin production (ng/ml) by macrophage-depleted non-adherent intestinal mononuclear cells cultured with or without autologous adherent cells (AC) from control and inflammatory bowel disease (IBD) mucosa

	Controls ($n=4$)		IBD ($n=9$)	
	without AC	with AC*	without AC	with AC
IgA	5436±1982	8204±2570	2112±1046	4085±2240†
IgG	224±80	836±460	811±214	2324±406†
IgM	333±136	568±163†	318±111	663±210†

* One million of AC were cultured with non-adherent intestinal mononuclear cells at 1×10^6/ml.
† $P<0.01$ (paired Student's *t*-test).

Table 4. Immunoglobulin production (ng/ml) by macrophage-depleted intestinal mononuclear cells by panning with 3C10 in presence or absence of autologous adherent cells (AC)

	Without AC	With AC*
IgA	1808±903	2553±934
IgG	943±628	2105±842
IgM	196±66	727±292

* One million AC were cultured with macrophage-depleted intestinal mononuclear cells at 1×10^6/ml, significance $P<0.01$ (Wilcoxon signed rank) for all immmunoglobulin isotypes.

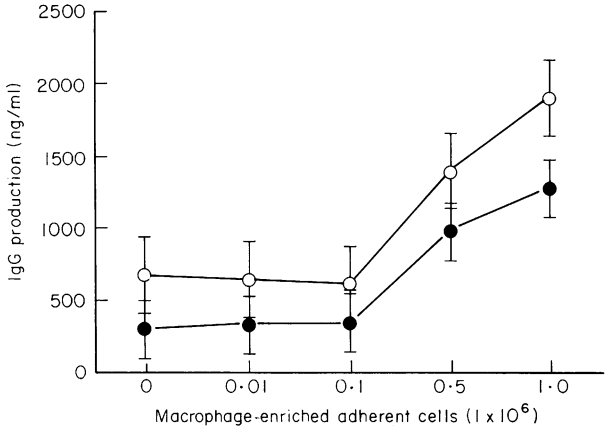

Fig. 3. IgG production by macrophage-depleted fibronectin non-adherent intestinal cells (○) ($n=7$) or cells after panning with antibody 3C10 (●) ($n=3$) when incubated with macrophage-enriched adherent cells.

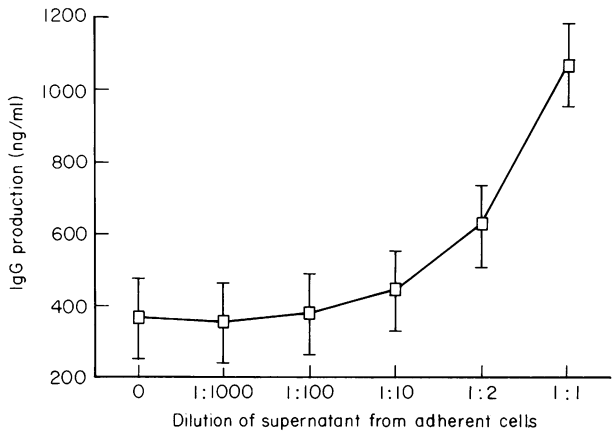

Fig. 5. IgG production by macrophage-depleted intestinal mononuclear cells cultured with different dilutions of supernatant from macrophage-enriched adherent cells. $n=4$.

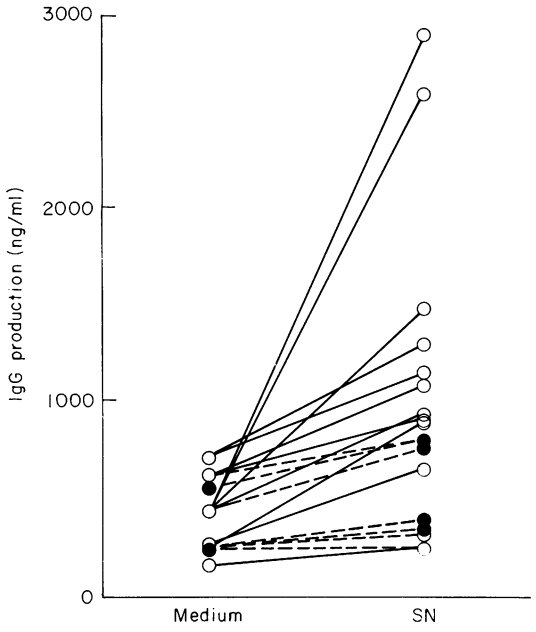

Fig. 4. IgG production by macrophage-depleted intestinal mononuclear cells in the presence or absence of supernatants (SN) derived from macrophage-enriched adherent cells isolated from control (●) ($n=7$) or inflammatory bowel disease mucosa (○) ($n=11$).

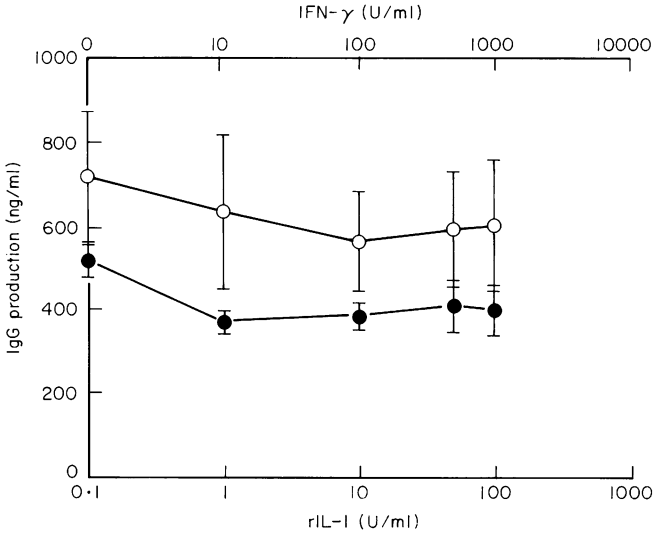

Fig. 6. IgG production by macrophage-depleted intestinal mononuclear cells in the presence of recombinant interleukin-1 (rIL-1) (○) or interferon-gamma (IFN-γ) (●).

Spontaneous production of IgA, IgG and IgM from intestinal MNC was significantly reduced following macrophage depletion by either fibronectin adherence or panning with 3C10 (Tables 1 and 2). This was seen in the control patients and patients with IBD. The reduction in immunoglobulin production by macrophage-depleted intestinal MNC after panning with 3C10 was greater in patients with IBD compared with control patients (Table 2). The percentage reduction in IgG produced by cells after panning with 3C10 strongly correlated ($r=0{\cdot}816$, $P<0{\cdot}001$) with the degree of macrophage depletion (Fig. 2). The reduction in immunoglobulin production following macrophage depletion, by either method, affected the three major immunoglobulin isotypes equally.

Effect of addition of macrophages on immunoglobulin production

There was a greater spontaneous production of IgA, IgG and IgM by both fibronectin non-adherent intestinal MNC (Table 3) and cells panned of macrophages by antibody 3C10 (Table 4) when incubated with autologous macrophage-enriched fibronectin-adherent cells. The increase in IgG production by fibronectin non-adherent cells and cells after panning with 3C10 was seen only when cultured with fibronectin-adherent cells at concentrations greater than 5×10^5/ml (Fig. 3).

Effect of supernatants from cultured macrophages on immunoglobulin production

Culture supernatants from macrophage-enriched fibronectin-adherent cells, isolated from control or IBD mucosa, were examined for their ability to stimulate immunoglobulin production by intestinal MNC depleted of macrophages by panning.

Table 5. Acid susceptibility of supernatant from macrophage-enriched adherent cells to enhance immunoglobulin production

	IgG (ng/ml) by panning* supplemented with			
Experiment	CM	AT-CM	SN	AT-SN
1	157	136	576	141
2	258	229	540	235
3	620	720	712	636

IgG values are the mean of triplicate observations.

* Macrophage-depleted intestinal mononuclear cells were cultured at 1×10^6/ml with 50% of supernatant.

CM, culture medium (10% fetal calf serum in RPMI); AT-CM, acid-treated culture medium; AT-SN, acid-treated supernatant.

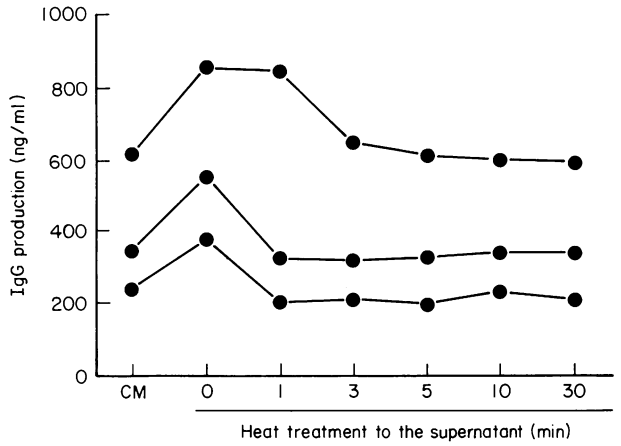

Fig. 7. IgG production by macrophage-depleted intestinal mononuclear cells cultured with the supernatants treated with heating for various periods. The supernatants were derived from 12-day cultures of macrophage-enriched adherent cells. CM, culture medium.

The immunoglobulin concentration initially present in the supernatants was measured and subsequently subtracted from the total. The supernatant immunoglobulin was always less than 10% of the total and was frequently undetectable.

Spontaneous IgG production by the macrophage-depleted intestinal MNC was enhanced by supplementing the culture medium with 50% (v/v) of supernatant from the cultures of macrophage-enriched fibronectin-adherent cells (Fig. 4). This increase in IgG produced by macrophage-depleted cells was dose dependent of supernatant (Fig. 5).

The percentage increase in IgG production by macrophage-depleted intestinal MNC incubated with supernatant from IBD mucosa (184+55%) was greater ($P<0{\cdot}05$) than that from control mucosa (24+9%).

Seven experiments on the effect of supernatant from cultured normal human peripheral blood monocytes on immunoglobulin production showed that there was no difference in IgG production between cells incubated with monocyte supernatants (450+74ng/ml) or with culture medium alone (453+70ng/ml).

Incubation with recombinant human IL-1 or IFN-γ had no effect on IgG production by macrophage-depleted intestinal MNC after panning with antibody 3C10 (Fig. 6).

Characteristics of supernatant from cultured macrophages

As seen in Table 5, the enhancing effect of the supernatant from macrophage-enriched intestinal MNC on IgG production by macrophage-depleted cells was abolished by treatment with acid pH 2 for 30 min. Heating also destroyed the activity of the supernatant (Fig. 7).

DISCUSSION

Macrophages are known to be cells of great morphological and functional heterogeneity. Very little is known of the functions of human intestinal macrophages. In this study, we investigated the effect of human intestinal macrophages and macrophage-derived soluble factor(s) on immunoglobulin production by isolated intestinal MNC.

To determine the influence of macrophages on immunoglobulin production by intestinal MNC, depletion by fibronectin adherence and the effect of adding back adherent cells was investigated. Depletion of adherent cells significantly reduced immunoglobulin production (Table 1), and addition of adherent cells to intestinal MNC enhanced immunoglobulin production (Table 3). These results indicate an up-regulatory effect on immunoglobulin production by human intestinal macrophages.

Intestinal macrophages are poorly adherent to glass or plastic surfaces, and the method to purify them has been greatly improved by coating the surface with plasma fibronectin (Winter *et al.*, 1983); nevertheless, immunological panning remains a better method of macrophage selection (Wysocki & Sato, 1978). Using panning with the macrophage-specific monoclonal antibody 3C10, depletion of macrophages from intestinal MNC was greater than using fibronectin adherence (Fig. 1). Depletion of 3C10 antibody-binding cells significantly reduced the immunoglobulin production by intestinal MNC (Table 2), which could be restored by the addition of fibronectin-adherent cells (Table 4). These findings confirm the results obtained using fibronectin adherence. It has been reported that mouse macrophages can enhance the level of antibody response (Inaba, Nakano & Muramatsu 1981; Inaba *et al.*, 1983). A recent study (Verspaget *et al.*, 1988) on immunoglobulin synthesis by intestinal MNC was in agreement with ours in that a fraction of the MNC depleted of macrophages by counter-flow elutriation centrifugation produced significantly less spontaneous IgA, IgG and IgM.

Macrophages secrete numerous products through which they exert various functions (Takemura & Werb, 1984). Macrophage down-regulatory activity for lymphocytes can be mediated by the release of soluble factors such as prostaglandin (Kennedy, Stobo & Goldyne, 1980; Brett, 1987) and reactive oxygen intermediates (Stenson & Parker, 1980). Immune enhancement by macrophages has been shown to be mediated by the secretion of IL-1 (Smith *et al.*, 1980) and IFNs (Maehara & Ho, 1977). Results of this study show that, in contrast to peripheral blood monocytes, intestinal macrophages release soluble factor(s) which enhance immunoglobulin production by intestinal MNC in a dose-dependent fashion (Fig. 5). The enhancing effect of the macrophage-derived supernatant is acid and heat labile (Table 5 and Fig. 7), and did not appear to be

either IL-1 or IFN-γ (Fig. 6). Whether other cytokines, such as IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), or combinations of these factors are responsible for the enhancing effect of macrophage-derived supernatant remains to be defined.

Patients with IBD have an altered pattern of spontaneous immunoglobulin production by isolated intestinal MNC (Bull & Bookman, 1977; MacDermott *et al.*, 1981; Wu *et al.*, 1989). Altered immunoregulation in IBD has been postulated to explain this phenomenon. However, it has been shown that patients with Crohn's disease have no alteration of immunoregulatory T cell function for immunoglobulin synthesis at the gut mucosal level (James *et al.*, 1985; Elson, Machelski & Weiserbs 1985). Verspaget *et al.* (1988) postulated that immunoglobulin production may be inhibited by activated macrophages in Crohn's disease. We have compared the effect of intestinal macrophages on immunoglobulin production in control subjects and in patients with IBD. The reduction of immunoglobulin production by macrophage-depleted intestinal MNC and the increase in immunoglobulin production by the cells supplemented with supernatant from adherent cell cultures were greater in patients with IBD. These results may indicate greater activation of intestinal macrophages from IBD. Evidence for such activation of the mononuclear phagocyte population has already been reported in patients with IBD (Doe *et al.*, 1980; Doe & Dorsman, 1982; Mahida *et al.*, submitted for publication), but whether these activated macrophages contribute to the pathogenesis of IBD remains unclear.

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