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Effect of Interferon-γ on Lymphocyte Cell Subsets in Human Large Bowel: A Study Using Organ Culture Method

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Suzuki, T., Horie, Y., Chiba, M., Iizuka, M. and Masamune, O. *Effect of Interferon-γ on Lymphocyte Cell Subsets in Human Large Bowel: A Study Using Organ Culture Method.* Tohoku J. Exp. Med., 1997, **181** (4), 431-446 —— This study was conducted to investigate the effects of interferon (IFN)-γ on normal colonic lamina propria lymphocyte subsets in humans using organ culture method. Lamina propria lymphocyte subsets in normal colonic biopsy tissues receiving 1×10^5 u/ml of IFN-γ (IFN-γ-treated group) were investigated in comparison with those cultured in medium only (IFN-γ-non-treated group) for 24 hr. CD8-positive cells and IgG, IgA1 and IgM-containing cells were elevated in the IFN-γ-treated group compared with those in the IFN-γ-non-treated group, which was similar to immunological changes in mucosal lesions of inflammatory bowel disease. ——
IFN-γ; large intestine; lymphocyte subsets; organ culture

It has been reported that the expression of HLA-DR antigens has never been found in normal human colonic epithelium (Pessara et al. 1988; Lundin et al. 1990; Ishii et al. 1994). However, the expression has been reported in colonic epithelium lesions by inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease, wherein even stronger expression at higher rates as observed (Selby et al. 1983; Iizuka et al. 1987, 1990; Chiba et al. 1994) as compared with other forms of colitis such as infectious colitis that are excluded from IBD.

Since antigen recognition by helper/inducer T cells can be performed in the presence of HLA-DR antigens (Pessara et al. 1988), it is speculated that the expression of HLA-DR antigens on colonic epithelium in IBD might be related to the progression and duration of the inflammation (Kuby 1992). The mechanism of expression of HLA-DR antigens on colonic epithelium has not been clarified, but it has been reported that interferon-γ (IFN-γ)-positive mononuclear cells increased on the lamina propria near HLA-DR antigen-positive colonic epithelium in IBD lesions (Matsumoto et al. 1989). It has also been observed that IFN-γproduced by the stimulation of mononuclear cells on lamina propria in IBD lesions induced HLA-DR antigens on colonic epithelium (Lowes et al. 1992) and

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increased IFN-γ has also been shown in IBD lesions (Fais et al. 1991; Lowes et al. 1992; Noguchi and Hiwatashi 1992; Sasaki et al. 1992). Therefore, it is speculated that IFN-γ plays an important role in HLA-DR antigen induction on colonic epithelium in IBD lesions. In fact, we have confirmed the existence of IFN-γ induced HLA-DR antigens at a remarkably high rate on normal human colonic epithelium using organ culture method (Ishii et al. 1994). In a series of studies of cytokines including IL-2, IL-4, IL-6, IL-8 and TNF-α, using the same organ culture method, IL-6 and IL-8 did not reveal inducibility of HLA-DR antigens, while TNF-α did by the mediation of IFN-γ. Moreover, IL-2 and IL-4 revealed inducibility of HLA-DR antigens, but the rate was less than 50% of all cases, which does not parallel the extent to which IFN-γ induced HLA-DR antigens on colonic epithelium (Horie et al. unpublished data). This suggests that IFN-γ is somehow intimately involved in the expression of HLA-DR antigens on colonic epithelium in IBD.

However, except for in our study of induction of HLA-DR antigen expression on human colonic epithelium, the effects of IFN-γ on intestinal mucosa have not been investigated at all. This study is aimed at clarifying the effects of IFN-γ on surface antigens on colonic mucosal lymphocytes in gut-associated lymphoid tissues using organ culture method.

Materials and Methods

Biopsy specimen

Three biopsy specimens (wet weight 4–12 mg/one specimen) taken from normal proctosigmoid of 16 patients with polyps (10 males, 6 females, average age: 64.5 years old), who underwent endoscopy of large intestine in our clinic, were used for this study. Prior to biopsy, informed consent was obtained from these patients.

Organ culture method

Of the three biopsy specimens taken from each patient, one was fixed immediately with PLP (periodate lysine-4% paraformaldehyde) and used as a control. The remaining two specimens were immediately organ-cultured by the method of Eastwood and Trier (1973). Briefly, specimens put on stainless mesh were placed on the liquid surface of the central well filled with 1,000 μl of culture medium in a culture dish (Falcon, Fraklin Lakes, NJ, USA). Then, it was cultured for 24 hr in an anaerobic incubator (Gaspak 100, Becton-Dickinson, Mountain View, CA, USA) filled with mixed gas of 95% O_2 and 5% CO_2 at 37°C. The specimens were laid on the stainless mesh with the epithelium side up and the section side down such that the bottom part of the specimen was in contact with the culture medium through the mesh.

As a culture medium, Trowell T8 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Gibco), 100 u/ml of penicillin G, 100

μg/ml of streptomycin and 5 μg/ml of gentamicin was used. Two kinds of specimens for each case, one cultured with and the other cultured without 1×10^5 u/ml of recombinant HuIFN-γ (Genzyme, Cambridge, MA, USA) were made. These were called the IFN-γ-treated group and the IFN-γ-non-treated group, respectively.

Identification of HLA-DR antigens on colonic epithelium and subsets of lamina propria lymphocytes

Identification of HLA-DR antigens on colonic epithelium and subsets of lamina propria lymphocytes in each specimen after organ culture were determined by indirect immunoperoxidase staining method according to Isobe's method (Isobe et al. 1977). Briefly, specimens obtained 24 hr after organ culture were fixed with PLP and then washed. Next, continuously frozen 6 μm wide sections were made.

Endogenous peroxidase activity was blocked with 50 mM periodic acid and 30 mM sodium borohydride.

Anti-human mouse monoclonal antibodies (Table 1) used as the first antibody were Nu-Ia (Matsumoto 1984: Nichirei, Tokyo), anti CD4 (Evans et al. 1981; Ledbetter et al. 1981: Boehringer Mannheim Biochemica, Mannheim, Germany), CD8 (Yano et al. 1989: Nichirei), CD5 (Wang et al. 1980), CD16 (Aparicio-Pages et al. 1989), IgG (Kuritani and Cooper 1982a), IgA1 (Conley et al. 1980), IgA2 (Conlay et al.1980), IgM (Kuritani and Cooper 1982a), IgD (Kuritani and Cooper 1982b: Becton-Dickinson), IgE (Sugi et al. 1984: Yamasa Shoyu, Choshi). Nu-Ia antibody recognizes HLA-DR antigens (Matsumoto 1984). Anti-CD4, CD8, CD5,

TABLE 1. *Monoclonal antibody*

Antibody	Company of product
HLA-DR antigen	
Nu-Ia	Nichirei, Tokyo
T lymphocyte	
pan T lymphocyte	
anti CD5 antibody	Becton-Dickinson, Mountain View, CA, USA
helper/inducer T lymphocyte	
anti CD4 antibody	Boeringer Mannheim, Mannheim, Germany
suppressor/cytotoxic T lymphocyte	
anti CD8 antibody	Nichirei
NK cell	
anti CD16 antibody	Becton-Dickinson
B lymphocyte	
anti IgA1 antibody	Becton-Dickinson
anti IgA2 antibody	Becton-Dickinson
anti IgM antibody	Becton-Dickinson
anti IgG antibody	Becton-Dickinson
anti IgD antibody	Becton-Dickinson
anti IgE antibody	Yamasa Shoyu, Choshi

and CD16 antibodies recognize helper/inducer T cells (Evans et al. 1981; Ledbetter et al. 1981), suppressor/cytotoxic T cells (Yano et al. 1989), pan T cells (Wang et al. 1980), and NK cells (Aparicio-Pages et al. 1989), respectively.

Sections were treated with the above antibodies in a moist chamber at room temperature for 30 min.

Next, they were treated with the second antibody, horseradish peroxidase-labeled rabbit anti-mouse immunoglobulins (Dako, Copenhagen, Denmark) in a moist chamber at room temperature for 30 min, and then colored with 3, 3′-diaminobenzidine 4HCl (DAB). To facilitate observation, the nucleus was stained with methylgreen solution. In addition, for the histological investigation, one section of each specimen was stained with hematoxylin and eosin (HE).

Expression of HLA-DR antigens on colonic epithelium and subsets of colonic lamina propria lymphocytes

Expression of HLA-DR antigens on colonic epithelium was determined under 400-fold magnification microscopy. Subset counting of colonic lamina propria lymphocytes was evaluated under 400-fold magnification microscopy and expressed as the number of positive cells per 1 mm^2 of lamina propria. The distribution of positive cells was often non-homogeneous even in the same section, and accordingly, the most well-balanced parts, except for extremely dense or sparse parts, were selected to be counted.

Statistical analysis

Results of subset counts of T lymphocytes and subset counts of B lymphocytes were statistically analyzed with the Wilcoxen test. p-Values less than 5% were considered to be significant.

Results

From morphological observation of sections stained with HE, colonic mucosa at 24 hr after culture showed histologically normal results.

In the control specimen and in the IFN-γ-non-treated group (Fig. 1a), expression of HLA-DR antigens in colonic epithelium was not observed. On the other hand, it was observed in all 16 specimens of the IFN-γ-treated group (Fig. 1b).

Subsets of colonic lamina propria T lymphocytes

CD4-positive cell counts on colonic lamina propria in the IFN-γ-treated group and in the IFN-γ-non-treated group were $141 \pm 28/mm^2$ and $100 \pm 33/mm^2$, respectively. CD8-positive cell counts were $226 \pm 42/mm^2$ and $119 \pm 24/mm^2$, respectively. CD5-positive cell counts were $416 \pm 72/mm^2$ and $306 \pm 54/mm^2$, respectively. The CD4/CD8 ratio was 0.88 ± 0.25 and 1.09 ± 0.16, respectively. CD 16-positive cell counts were $85 \pm 15/mm^2$ and $65 \pm 13/mm^2$, respectively (Table 2, Fig. 2). The IFN-γ-treated group showed a statistically significant increase

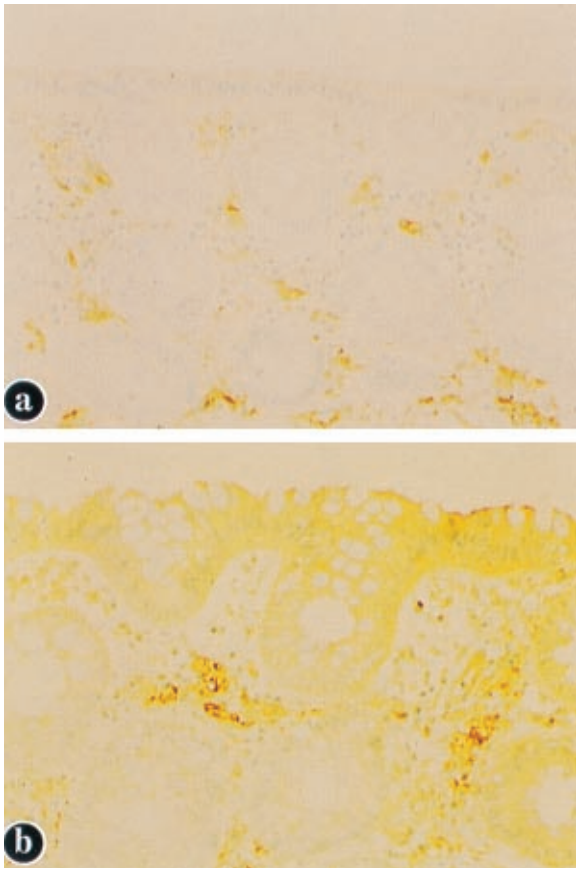

Fig. 1. Epithelial HLA-DR antigen.
a, Interferon-γ-non-treated group ($\times 400$): Epithelial HLA-DR antigen is negative.
b, Interferon-γ-treated group ($\times 400$): Epithelial HLA-DR antigen is positive.

TABLE 2. *T lymphocyte subset in colonic lamina propria*

	Interferon-γ treated group ($n=16$)		Interferon-γ non-treated group ($n=16$)
CD4/mm^2	141±28		100±33
CD8/mm^2	226±42	**	119±24
CD5/mm^2	416±72		306±54
CD16/mm^2	85±15		65±13
CD4/CD8	0.88±0.25		1.09±0.16

(Mean±S.E.) $^{**}p<0.01$

only in CD8-positive cell counts as compared with the IFN-γ-non-treated group (Figs. 2, 3). Moreover, the IFN-γ-treated group showed an increased tendency in CD4-positive cell counts and in CD5-positive cell counts. However, a decreased tendency in the CD4/CD8 ratio was observed in the IFN-γ-treated group while CD16-positive cell counts did not show any difference between the two groups.

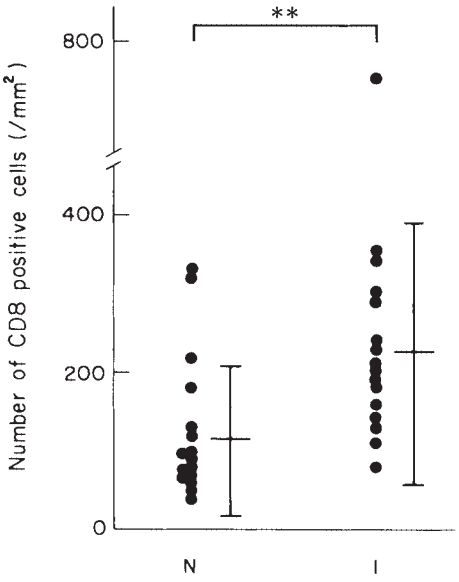

Fig. 2. The number of CD8 positive cells.
N stands for Interferon-γ-non-treated group and I stands for Interferon-γ-treated group.
Three horizontal bars mean $\pm$ S.E. $^{**}p<0.01$.

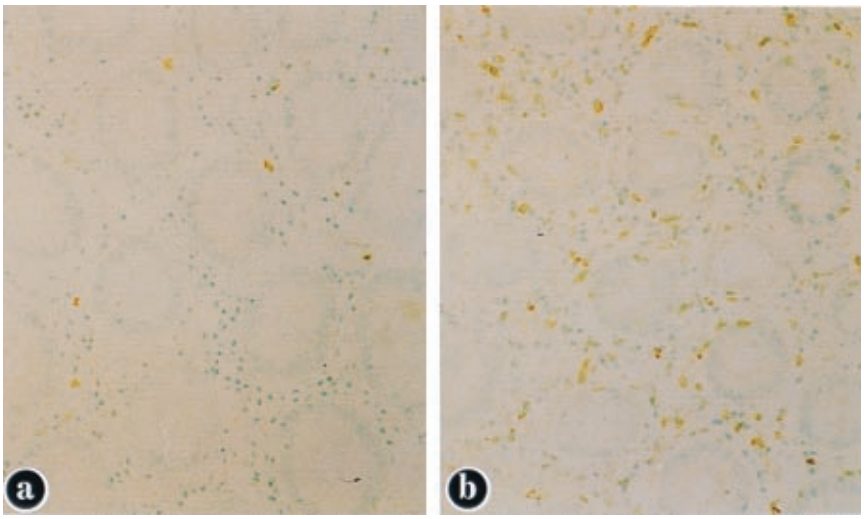

Fig. 3. CD8 positive cells.
a, Interferon-γ-non-treated group. ($\times$400)
b, Interferon-γ-treated group. ($\times$400)

Subsets of colonic lamina propria B lymphocytes

IgG-containing cell counts on colonic lamina propria in the IFN-γ-treated group and in the IFN-γ-non-treated group were $222\pm39/mm^2$ and $104\pm18/mm^2$, respectively. IgA1-containing cell counts were $516\pm51/mm^2$ and $401\pm47/mm^2$, IgA2-containing cell counts were $561\pm88/mm^2$ and $446\pm66/mm^2$, IgM-containing cell counts were $194\pm28/mm^2$ and $130\pm15/mm^2$, IgD-containing cell counts were $68\pm21/mm^2$ and $38\pm10/mm^2$, IgE-containing cell counts were $218\pm50/mm^2$ and

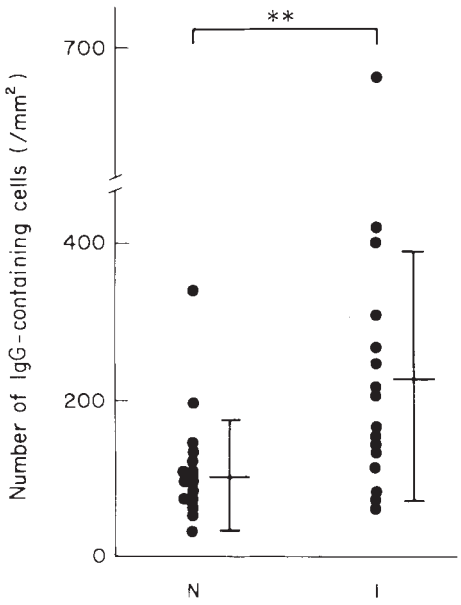

Fig. 4. The number of IgG-containing cells.
N stands for Interferon-γ-non-treated group and I stands for Interferon-γ-treated group.
Three horizontal bars mean$\pm$S.E. $^{**}p<0.01$.

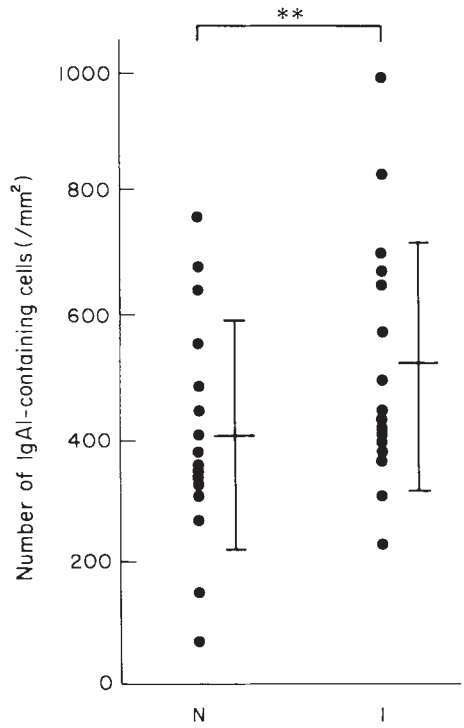

Fig. 5. The number of IgA1-containing cells.
N stands for Interferon-γ-non-treated group and I stands for Interferon-γ-treated group.
Three horizontal bars mean$\pm$S.E. $^{**}p<0.01$.

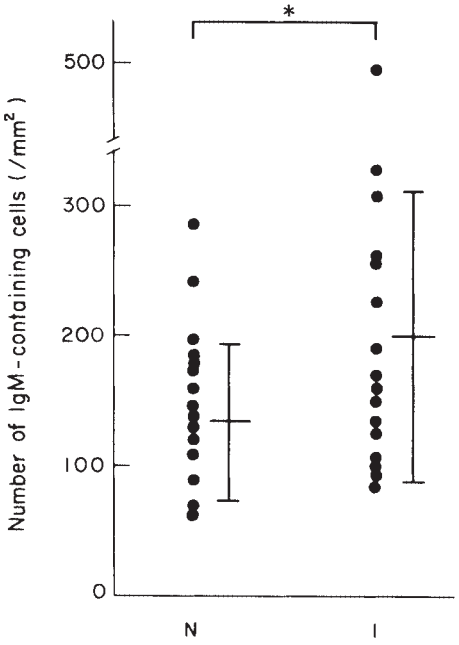

Fig. 6. The number of IgM-containing cells.
N stands for Interferon-γ-non-treated group and I stands for Interferon-γ-treated group.
Three horizontal bars mean $\pm$ S.E. $^{*}p<0.05$.

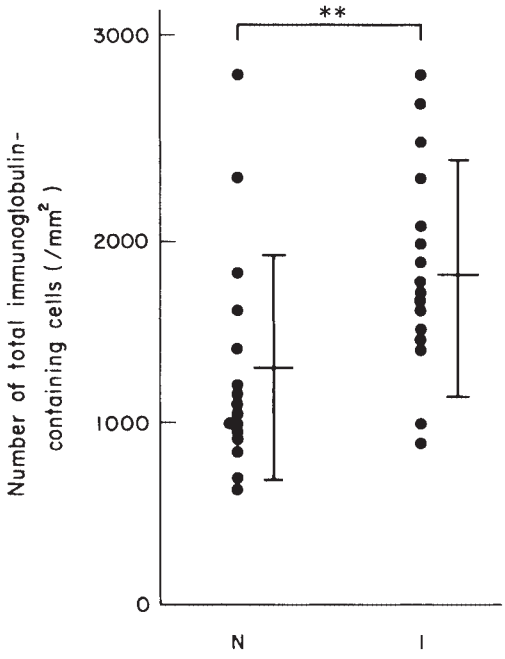

Fig. 7. The number of total immunoglobulin-containing cells.
N stands for Interferon-γ-non-treated group and I stands for Interferon-γ-treated group.
Three horizontal bars mean $\pm$ S.E. $^{**}p<0.01$.

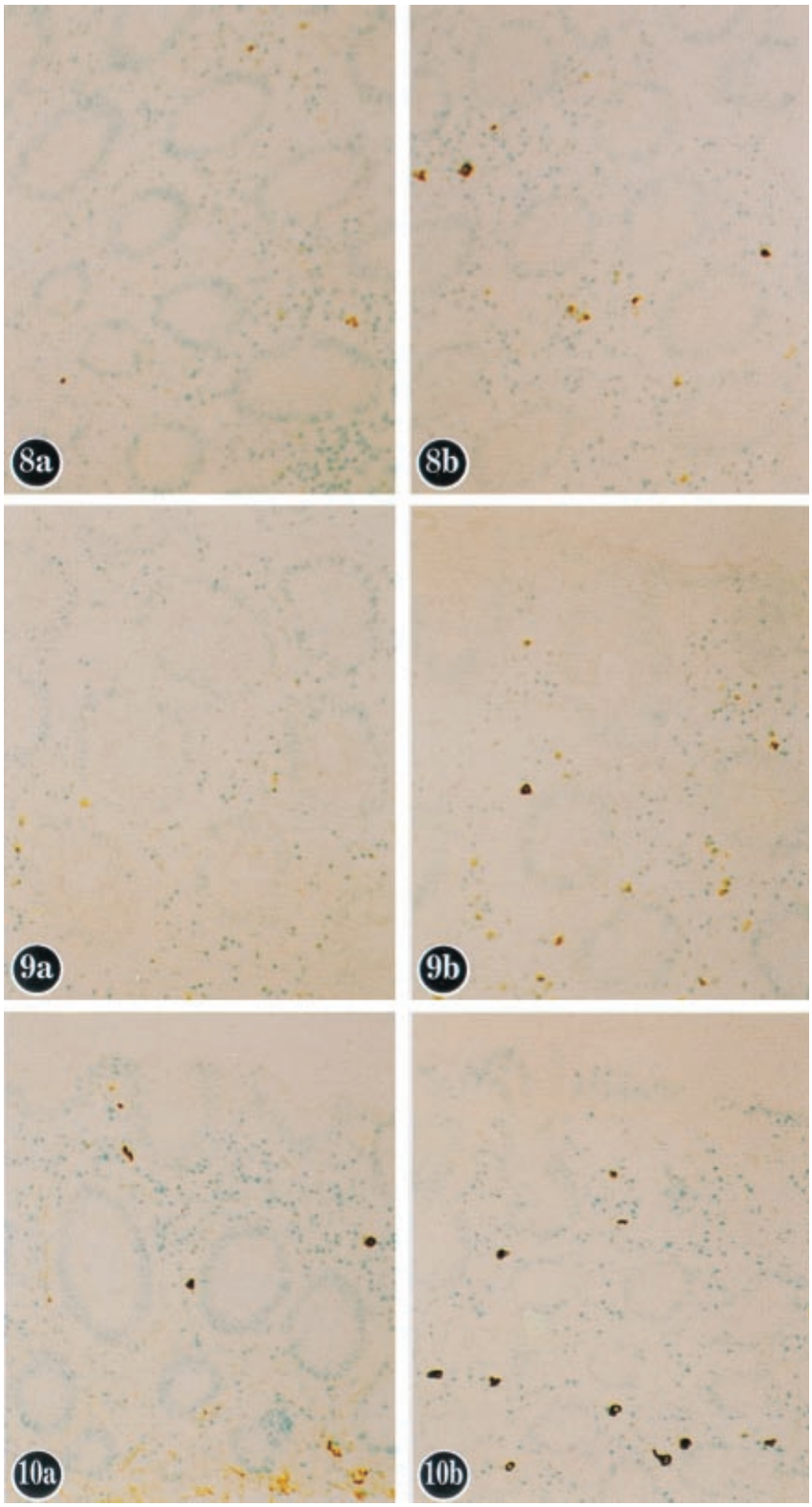

Fig. 8. IgG-containing cells.
a, Interferon-γ-non-treated group. ($\times$400)
b, Interferon-γ-treated group. ($\times$400)
Fig. 9. IgA1-containing cells.
a, Interferon-γ-non-treated group. ($\times$400)
b, Interferon-γ-treated group. ($\times$400)
Fig. 10. IgM-containing cells.
a, Interferon-γ-non-treated group. ($\times$400)
b, Interferon-γ-treated group. ($\times$400)

TABLE 3. *B lymphocyte subset in colonic lamina propria*

	Interferon-γ treated group ($n=16$)		Interferon-γ non-treated group ($n=16$)
IgG/mm^2	222±39	**	104±18
IgA1/mm^2	516±51	**	401±47
IgA2/mm^2	561±88		446±66
IgM/mm^2	194±28	*	130±15
IgD/mm^2	68±21		38±10
IgE/mm^2	218±50		277±54
Total B/mm^2	1,765±151	**	1,306±147

(Mean±S.E.) $^*p<0.05$ $^{**}p<0.01$

277±54/mm^2 and total immunoglobulin-containing cell counts including all immunoglobulin cell counts were 1765±151/mm^2 and 1306±147/mm^2, respectively (Figs. 4, 5, 6, 7, Table 3). The IFN-γ-treated group showed an increase in IgG-containing cell counts (Figs. 4, 8), an increase in IgA1-containing cell counts (Figs. 5, 9) and in IgM-containing cell counts (Figs. 6, 10) compared with the IFN-γ-non-treated group. Consequently, total immunoglobulin cell counts in the IFN-γ-treated group also increased with statistical significance (Fig. 7).

Moreover, the IFN-γ-treated group showed an increased tendency in IgD-containing cell counts. However, IgA2-containing cell counts and IgE-containing cell counts did not show any difference between the two groups (Table 3).

DISCUSSION

In this study, we reconfirmed the results presented by Ishii et al. (1994) that HLA-DR antigen expression on human colonic epithelium was induced by IFN-γ.

It is well known that IFN-γ promotes differentiation of cytotoxic T cells (Maraskovsky et al. 1989) and modulates proliferation and differentiation of helper/inducer T cells (Cherwinski et al. 1987; Belosevic et al. 1989; Scott 1991) among T lymphocytes while increasing immunoglobulin containing cells (Leibson et al. 1984; Sidman et al. 1984; Defrance et al. 1986; Snapper et al. 1987; Nakagawa et al. 1988; Arai and Kuwano 1991) other than IgE among B lymphocytes. However, the effects of IFN-γ on immunocompetent cells of the intestinal lymphoid tissues have not been investigated at all.

In the organ culture method, tissues have been maintained in the same conditions as in vivo. Therefore, this method can be considered as an experimental model to evaluate the effects of IFN-γ on intestinal lymphoid tissues under physiological conditions. In other words, this method can be used to assess the reflection of cytokine cascades on the intestinal mucosa as well as to know the effects of IFN-γ, either directly or indirectly mediated through HLA-DR antigens

induced on colonic epithelium, on the lamina propria mononuclear cells.

Ouyang et al. (1988) have reported that IFN-γ concentration on normal intestinal mucosa was $0.9\text{-}1.2\times10^2$ u/ml. Considering the extremely short half-life of cytokines, further increase in IFN-γ concentration might be expected in IFN-γ-releasing sites and in inflammatory sites. Moreover, studies performed by Ishii et al. (1994) and Horie et al. (in preparation) using the same experimental method as this one showed the expression of HLA-DR antigens initiated at 1×10^2 u/ml of IFN-γ concentration and reaching up to 100% expression at 1×10^5 u/ml of IFN-γ concentration on colonic epithelium. Consequently, 1×10^5 u/ml of IFN-γ concentration was added into the organ culture medium in this study.

T lymphocytes

This study showed that IFN-γ increased CD4-positive cells and CD8-positive cells on the intestinal lamina propria. Though effects of IFN-γ on T lymphocytes had already been reported (Cherwinski et al. 1987; Belosevic et al. 1989; Maraskovsky et al. 1989; Scott 1991), this study made it clear for the first time that IFN-γ modulated T lymphocytes on the colonic lamina propria as well.

In this experimental model, since the tissues were separated from the blood and lymph circulatory systems, there is no possibility that increase of T lymphocytes was caused by the migration of T lymphocytes from the host circulatory system itself. Therefore, it might be considered that IFN-γ directly promoted differentiation and induction of surface antigens on lymphocytes and then increased CD4-positive cells and CD8-positive cells. As a possible mechanism of increase of CD8-positive cells, HLA-DR antigens induced on colonic epithelium might stimulate helper/inducer (CD4-positive) T cells, which might have led to the increase in suppressor/cytotoxic (CD8-positive) T cells.

Furthermore, through the cytokine cascade existing on the colonic mucosa, the addition of IFN-γ might be thought to induce IL-2 and TNF-α from Th1 cells (Cherwinski et al. 1987). It has been reported that IL-2 induced cytotoxic T cells in cooperation with IFN-γ(Maraskovsky et al. 1989) and TNF-α promoted the division of T cells (Kohase 1990). Therefore, through the mediation of cytokine cascade, IFN-γ might be considered to contribute to the increase of CD4 and CD8-positive cells.

For further analysis of the findings in more detail, the concentration of IL-2 and TNF-α existing on colonic mucosa as well as the quantitative changes of T lymphocyte subsets including Th1 and Th2 cells on the lamina propria should be considered for investigation.

There have been several reports (Selby et al. 1984; Yuan et al. 1984; Kobayashi et al. 1988; Hayashi 1989; Senju et al. 1990) regarding the changes of T lymphocyte subsets on intestinal lamina proria, however, conclusions have yet to be obtained. Results obtained from this study were consistent with several reports that CD8-positive cells increased on the lamina propria lesions in Crohn's

disease (Yuan et al. 1984; Senju et al. 1990). Therefore, it is suggested that IFN-γ plays an important role in modulating T-lymphocytes on the lamina propria lesions in Crohn's disease.

Immunoglobulin-containing cells

This study revealed that IFN-γ showed a statistically significant increase in IgA1-, IgM- and IgG-containing cells and a tendency to increase IgD-containing cells on the intestinal lamina propria, which resulted in an increase of the total immunoglobulin-containing cells. Supplementing other reports on the effects of IFN-γ on immunoglobulin-containing cells, this study demonstrated that IFN-γ modulated immunoglobulin-containing cells on colonic lamina propria as well.

It has yet to be clarified whether these changes were due to a direct effect of IFN-γ which promoted differentiation and proliferation of immunoglobulin-containing cells, or due to an indirect effect of IFN-γ mediating the activation of helper T cells (CD4-positive) on immunoglobulin-containing cells. For further investigation regarding the participation of helper T cells, an experimental model using anti-CD antibodies as a blocking agent would be required.

IFN-γ may be involved in the stimulation of the cytokine cascade which induces IL-2 and TNF-α from Th1 cells. It has been reported that IL-2 promotes the production of IgG1, IgG2a and IgM in cooperation with IFN-γ (Snapper and Paul 1987; Arai and Kuwano 1991), while simultaneously, induced TNF-α promoted the division of B cells as well as an increased antibody production (Kohase 1990). Therefore, IFN-γ might be considered to contribute to the increase of IgG-, IgM- and IgA1-containing cell counts in this study. It has been reported that such immunoglobulin-containing cells increased on mucosal lesions in ulcerative colitis or Crohn's disease (Brandtzaeg et al. 1974; Rosenkrans et al. 1980; Ohta 1986). This study suggests that IFN-γ is one of the factors participating in the increase of immunoglobulin containing cells on mucosal lesions in IBD.

However, IgG- and IgM-containing cells on the lamina propria lesions in ulcer ative colitis or Crohn's disease increased 5–30 fold higher than those of the normal lamina propria (Brandtzaeg et al. 1974; Rosenkrans et al. 1980; Ohta 1986). The results here also revealed wide difference between the IFN-γ-treated group and the IFN-γ-non-treated group in this respect. There are two possible explanations for this. The organ culture method is a method wherein the samples are separated from the lymph and blood circulatory systems and not only IFN-γ, but other cytokines such as IL-2, IL-4 and IL-6 each (Snapper et al. 1987; Schoele et al. 1991; Kitamura 1993) which promote B lymphocyte proliferation participated in changes on mucosal lesions in IBD.

Moreover, as stated above, changes in T and B lymphocytes stimulated by IFN-γ were very similar to those observed on mucosal lesions in IBD, suggesting that IFN-γ might be involved in the pathophysiological aspects of IBD.

The actual numbers of lymphocyte subsets per unit area after the organ culture in our present study decreased compared with those of our previous results before the organ culture. This may be attributed to a decline in the viability of lymphocytes in the lamina propria after the organ culture(Ohta 1986; Ohta et al. 1986). The degree of this decline differed from one lymphocyte subset to another and was particularly prominent in T lymphocyte. Therefore, the lymphocyte subsets after organ culture without IFN-γ, showed changes in their ratios compared with those without organ culture: a decline in the percentage of T lymphocyte of the total Ig-containing cell (14.4%) and an increase in the IgG-containing cell (7.9%) (Ohta 1986; Ohta et al. 1986; Brandtzaeg et al. 1987). The organ culture of the intestine has been studied by the viability of the epithelium (Eastwood and Trier. 1973). This is an established method which keeps epithelium viable after 24 hr of organ culture. But no study has been made of the changes in the viability of lymphocyte subsets in the lamina propria. Basic studies in this area are needed.

Conclusion

In this study, it was clarified that IFN-γ increased CD4-, CD8-positive cells, IgG-, IgA1-, IgM- and IgD-containing cells and total immunoglobulin containing cells on normal colonic mucosa in humans using the organ culture method.

Similar changes in T lymphocytes and B lymphocytes on mucosal lesions in IBD have been reported, suggesting that IFN-γ might be one of the factors involved in the changes of lymphocyte subsets on mucosal lesions in IBD.

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