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Immunobiological study of interferon-gamma-producing cells after staphylococcal enterotoxin B stimulation

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SUMMARY

Staphylococcal enterotoxin B (SEB) induced the production of human interferon-gamma (hIFN-γ) in peripheral blood mononuclear cells (PBMC). Using specific mouse monoclonal antibodies (mAb) to hIFN-γ, the patterns of cytoplasmic fluorescence in the PBMC from five individuals were studied. Discrete polar bodies in a ring-formation adjacent to the nuclear membrane was the most frequently observed fluorescent pattern throughout the 76-hr observation period. Additional and different fluorescent patterns such as multifocal and diffused cytoplasmic, as well as granular fluorescence over the whole cytoplasm, may appear during the late induction period (50–76 hr). By using an immunogold–silver (IGS) enhancement method to label cell-surface antigens, it was possible to detect the presence of CD3, CD4, CD25 and OKT11 marker in 55%, 54%, 77%, and 71% of the IFN-γ producer cells, respectively. Monensin and carboxylcyanide m-chlorophenyl-hydrozone (CCCP) are ionophores known to interrupt subcellular transport of a number of secretory proteins. When monensin or CCCP was added to the induced cultures 2–3 hr before harvests, an increase in the intensity of cytoplasmic fluorescence in IFN-γ-producing cells was observed; a greater than 10-fold enhancement in the sensitivity of immunostaining was demonstrated in these preparations.

INTRODUCTION

Human interferon-gamma (hIFN-γ) is a lymphokine secreted by cells in response to many mitogenic and immunogenic stimulants (Kirchner & Marcucci, 1984). This cellular protein is known to play a key role in the regulation of the body's immune response (Epstein, 1977; Sonnefeld & Merigan, 1979). It is also believed to be involved in the control of cell growth and differentiation (Taylor-Papadimitriou & Rozengurt, 1985). The ability to precisely determine the functional identity of IFN-γ producer cells that are responding to a particular inducing agent or circumstance is important for both clinical as well as theoretical reasons. Recent conventional approaches to this identity problem have been based primarily on the study of peripheral blood mononuclear cell (PBMC) populations depleted or augmented in certain subsets of cells by depletion or enrichment techniques; methods which are complex and time-consuming and of suspect effectiveness (Kirchner & Marcucci, 1984). The availability of highly specific polyclonal as well as monoclonal anti-IFN antibodies in recent years has led to the development of more direct means to identify IFN producer cells. Using very potent polyclonal and monoclonal antibodies (mAb) directed against hIFN-α, Timonen *et al.* (1980) and Saksela *et al.* (1984) were able to demonstrate by indirect immunofluorescent staining the presence of intracytoplasmic IFN-α in single peripheral blood cells. Cytoplasmic detection of hIFN-γ in anti-OKT3 antibody or phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) has also been reported recently (Andersson *et al.*, 1986; Laskay *et al.*, 1986). The cytoplasmic fluorescence indicative of IFN-γ appeared to localize in a polar juxtanuclear position coincident with the region of the Golgi apparatus.

In this communication data are presented on the induction of intracellular IFN-γ in SEB-treated human PBMC. The use of catabolic ionophores is introduced to enhance the detection of cytoplasmic IFN-γ with mAb. Furthermore, simultaneous visualization of IFN-γ and surface marker of the producer cell is demonstrated by a combined use of indirect immunofluorescence and immunogold–silver (IGS) staining procedures.

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MATERIAL AND METHODS

Preparation of PBMC and IFN-γ induction

Peripheral blood was collected from normal healthy donors in heparinized tubes (Becton-Dickinson, Rutherford, NJ). PBMC were isolated by Ficoll–Hypaque (Cederlane, Hornby, Ontario) gradient centrifugation (Lee & Epstein, 1980), washed twice in Hank's balanced salt solution and resuspended in RPMI-1640

(Flow Laboratories, Mississauga, Ontario) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 200 U/ml penicillin, 50 μg/ml streptomycin (growth medum). 0·1 ml of cell suspension containing $2·5 \times 10^6$ cells per ml was distributed to each microwell in a 96-well plate (Costar, Cambridge, MA). Another 0·1 ml growth medium containing 25 μg/ml ·SEB (Sigma Chemical Co., St Louis, MO) was added to induce IFN-γ. PBMC serving as control were cultured in growth medium alone. All cultures were incubated at 37° in a humidified 5% CO_2 incubator.

Stock solutions of monensin and carboxylcyanide m-chlorophenyl-hydrozone (CCCP; Sigma) were made up in absolute ethanol at a concentration of 10 mM/ml. In some experiments monensin or CCCP was added to the cultured PBMC (2–3 μM/ml final concentration) 2–3 hr immediately before the cells were collected for immunostaining.

Monoclonal antibodies (mAb)

The synthesis and cloning of the entire coding sequence of hIFN-γ and the efficient expression as authentic polypeptide by plasmid vector pJP_1R_3 in *Escherichia coli* and the characterization of the recombinant hIFN-γ have been described previously (Jay *et al.*, 1984a, b). The development of mouse mAb against the recombinant hIFN-γ has also been described previously (Alfa, Dembinski & Jay, 1987). The two mAb, mIF-3070 and mIF-3055, selected for the present study were of IgG1 isotype and were produced as ascites fluid in BALB/c mice; mIF-3070 has $2·0 \times 10^5$ neutralizing U/ml and $6·4 \times 10^5$ ELISA U/ml and mIF-3055 has $2·0 \times 10^5$ neutralizing and ELISA U/ml. The production, characterization and titration assay of these mAb have been reported earlier (Alfa *et al.*, 1987).

Immunofluorescent staining for cytoplasmic IFN-γ

Cultured PBMC were collected and washed once in phosphate-buffered saline (PBS) and spun onto precleaned slides (Shandon cytocentrifuge). The cytospin preparations were fixed with 3·5% paraformaldehyde in 0·1 M PBS and 0·2% NaCl for 30 min, rinsed and kept in PBS until stained (Saksela *et al.*, 1984). Immediately prior to staining, slides were transferred to a 0·05% solution of Nonidet P-40 for 15 min and washed three times with PBS. In some cases preparations were then treated with a 10% goat serum (Gibco/BRL, Burlington, Ontario)-PBS or 1% bovine serum albumin (BSA) in PBS (BSA-PBS) for 30 min. mAb in BSA-PBS were then applied to the specimens for 40 min at room temperature. After two rinses in PBS, the preparations were stained for 40 min with FITC-coupled $(Fab')_2$ fragment goat anti-mouse IgG conjugate (Cappel Product, Organon Technika, Scarborough, Ontario) diluted 1:50 with BSA-PBS. Non-induced PBMC cultures and specimens treated with labelled secondary antibodies alone were used as controls. All slides were washed twice with 0·05% Tween 20-PBS and once with PBS before mounted in glycerol phosphate buffer, pH 9·4 (Microtrak, Syva Co., Palo Alto, CA).

Immunogold–silver staining for cell-surface antigen

Approximately 2×10^6 cultured PBMC were aliquoted into a polystyrene centrifuge tube (Fisher Scientific, Louisville, KY; 1-ml capacity), and after two washes in BSA-PBS the cells were treated with mAb directed against leucocyte cell-surface antigen. The antibodies used included OKT11, OKB1 (Ortho Diagnostic System Inc., Don Mills, Ontario), CD3, CD4 and CD25 (Becton-Dickinson, Mountain View, CA). Each aliquot was treated with 10 μl of the OK-series antibodies (diluted 1:1 with BSA-PBS supplemented with 0·2% sodium azide) or 25 μl of the CD-series antibodies (undiluted) and incubated for 20 min at 4–8°. After three rinses in BSA-PBS supplemented with 0·2% azide, the cells were resuspended in 20 μl of colloidal gold (10 nm)-labelled goat anti-mouse antibodies (Jansen Life Science Product, Beerse, Belgium) for 20 min at 4–8°. The gold conjugates were diluted 1:50 with BSA-PBS-0·2% azide. Following three washes in BSA-PBS, the cells were spun onto precleaned slides (Shandon cytocentrifuge), fixed with 3·5% paraformaldehyde for 1 hr and stained for cytoplasmic IFN-γ according to the procedures described. Following two rinses in 0·05% Tween 20-PBS, the slides were washed three times in deionized distilled water, stained with the Silver Enhancer solution (the Intense Kit, Jansen Life Science Product) for 10–15 min, rinsed twice in distilled water and mounted in glycerol phosphate buffer, pH 9·4 (Microtrak).

Microscopy

A Nikon Optiphot 'X' Research microscope was used in this study. A total of 1000 cells was read from each specimen. Cells per randomly selected area were first enumerated using a 40 × objective lens under phase-contrast. The same area was scanned for positive FITC-stained cells using a MEB filter block for B_1 (Blue, 495 nm) excited epifluorescence. Combined epifluorescence and transmitted bright field illumination were used in the demonstration of IFN-γ and colloidal gold–silver-labelled cell-surface markers simultaneously.

IFN assay and characterization

IFN activity was assessed by the plaque-reduction method in human A549 cells using EMC virus as the challenge virus (Sonnefeld & Merigan, 1979). The NIH hIFN-γ reference standard (Gg23-901-530) used to calibrate the assay was provided by NIAID, Bethesda, MD. IFN titre was expressed as international units (IU) per ml. To identify hIFN-γ, aliquots of the test sample were incubated for 1 hr at room temperature with an appropriate dilution of rabbit polyclonal anti-hIFN-α antibody (Interferon Sciences, Inc., New Brunswick, NJ), rabbit anti-hIFN-β antibody (Lee Biomolecular Research Inc., San Diego, CA) and mouse mAb against hIFN-γ (Interferon Sciences), respectively, before being assayed for residual antiviral activity.

RESULTS

Pattern of cytoplasmic fluorescence

Two mouse mAb, mIF-3005 and mIF-3070 (diluted 1:100 in BSA-PBS), were used to detect the presence of cytoplasmic IFN-γ in SEB-treated PBMC from five normal individuals (aged 22–50 years). The pattern of cytoplasmic fluorescence appeared to be similar in all subjects, although the relative number of fluorescent cells varied somewhat from one individual to another. As early as 3 hr after stimulation, cytoplasmic fluorescence was detectable in 0·2–0·5% of PBMC. Fluorescence appeared as small discrete polar bodies arranged in a ring-formation adjacent to the nuclear membrane. This fluorescence became more intense over the next 24 hr. The percentage of fluorescent cells increased to 3·5–6% and peaked around 44–56

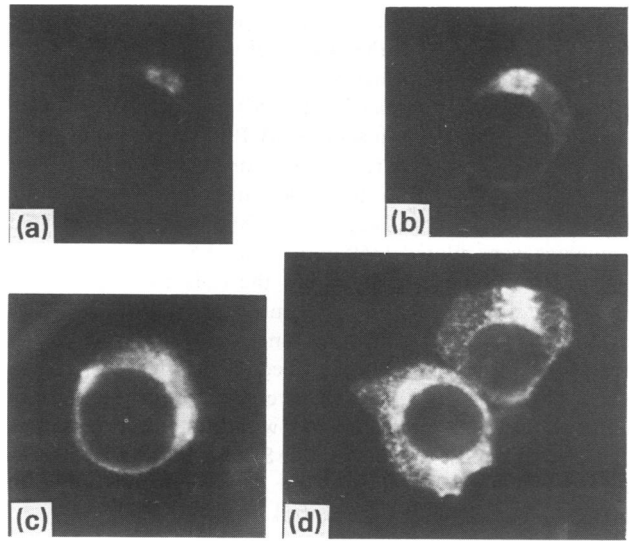

Figure 1. Patterns of cytoplasmic fluorescence in SEB-stimulated human PBMC. Positively stained polar bodies aligned in a ring-formation adjacent to the nuclear membrane was the most frequent fluorescent pattern throughout the 76 hr induction (a, b) (×650). Additional fluorescent patterns, such as multifocal, diffused as well as granular fluorescence, appeared during the latter part of the induction period (c, d) (×650).

hr. By 50-76 hr, this percentage fell slightly to 2·5–5%. The percentage of fluorescent cells at various intervals was based on 1000 cells read. During the late induction period (50–76 hr), additional and different fluorescent patterns, such as juxtanuclear multifocal and diffused cytoplasmic as well as granular fluorescence over the whole cytoplasm, appeared (Fig. 1).

No cytoplasmic fluorescence was detected in control uninduced specimens or in specimens treated with FITC conjugates or 10% goat serum alone. The number of positively stained cells in control cultures from all five subjects was less than 0·001%. In the control culture of one individual, after 16 hr culture, some cells (0·3%) appeared with relatively weak juxtanuclear ring-formation cytoplasmic fluorescence.

Enhancing effects of monensin and CCCP

Addition of monensin and CCCP to SEB-treated PBMC 2–3 hr before harvest for immunostaining enhances the intensity of cytoplasmic fluorescence (Fig. 2) This was true irrespective of whether the comparisons were made in cultures of 24, 48 or 65 hr post-SEB induction. Optimal concentrations of the two ionophores used were 2–4 μM/ml culture fluid. Concentrations beyond this range resulted in severe swelling and distortion of the cell's morphology. At lower concentrations (approximately 1 μM/ml, monensin and CCCP could be left in the culture media for more than 24 hr without seriously compromising the cell's architecture.

The sensitivity of immunostaining to monensin or CCCP treated and non-treated specimens was compared by using various dilutions of mIF-3055 and mIF-3070. Dilutions of these primary antibodies, which allowed the detection of cytoplasmic fluorescence in reactive cells, were 1:10,000, 1:4000 and 1:400 for monensin-treated, CCCP-treated and non-treated cells, respectively.

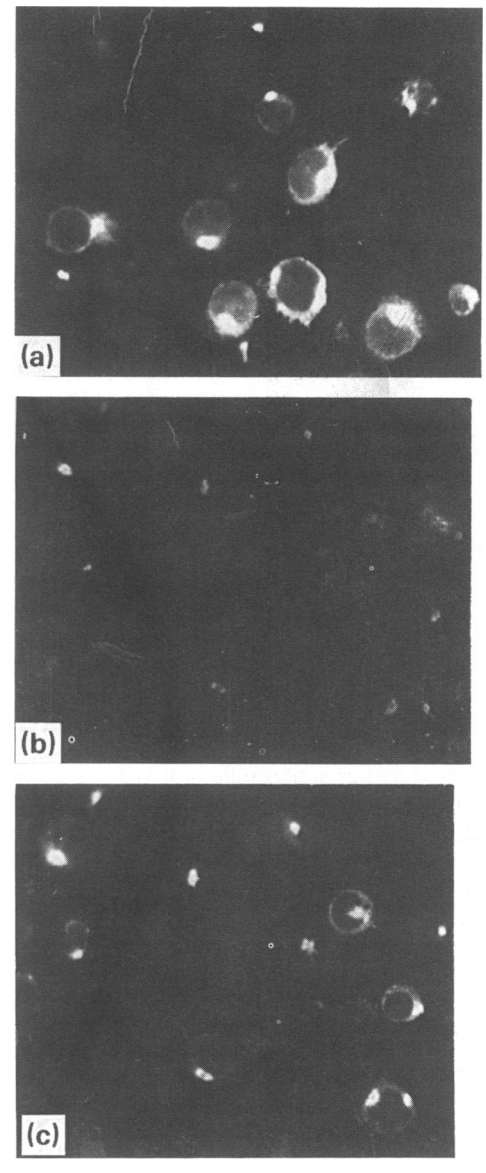

Figure 2. Cytoplasmic fluorescence in IFN-γ-induced PBMC: a visual comparison between the immunostaining intensity of monensin- and non-monensin-treated specimens. (a) Monensin-treated IFN-γ induced PBMC (48 hr culture). Picture taken with automatic light-sensor, timed 9 seconds. (b, c) Non-monensin-treated IFN-γ-induced PBMC (48 hr culture). Picture taken manually, 9 seconds exposure (b) and automatically, 25 seconds exposure (c). All three pictures were printed under indentical conditions, ×225.

As previously mentioned, one of the five subjects used in this study was found to have a small number of weakly stained cells in the controls that had been cultured for more than 16 hr. An enhancement in immunostaining was also observed in the samples when monensin or CCCP was added. The four individuals whose control PBMC specimens were completely negative to immunostaining remained so after the addition of monensin or CCCP. Cytoplasmic fluorescence was not observed in monensin- or CCCP-supplemented samples (SEB-induced or non-induced PBMC) that were stained with FITC conjugate alone.

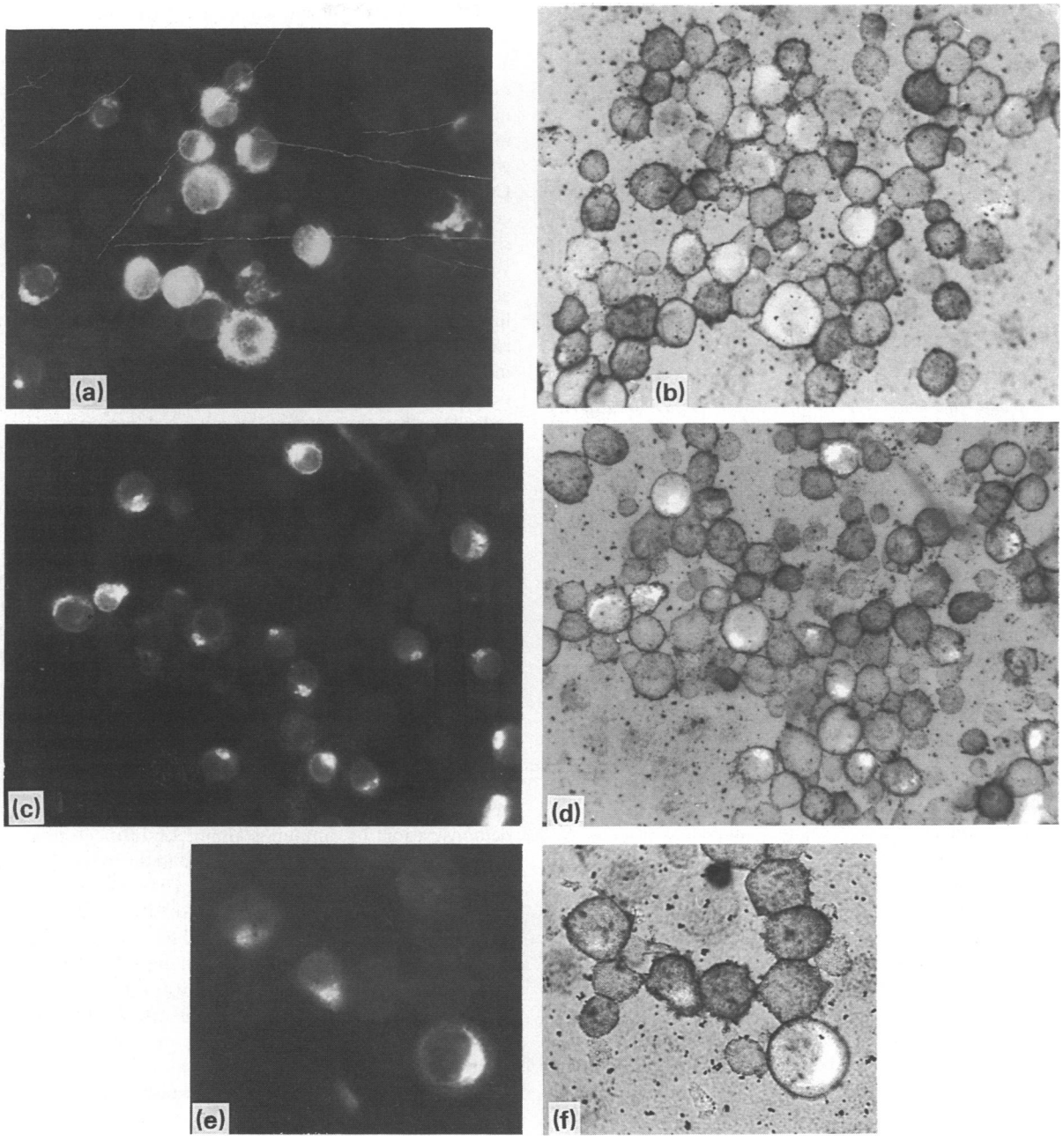

Figure 3. Double-staining of IFN-γ-producing cells. Simultaneous demonstration of cytoplasmic IFN-γ by FITC-labelled secondary antibodies and OKT11 (a, b), CD4 (c, d) and CD3 (e, f) cell surface markers by IGS-labelled antibodies in human PBMC. Pictures taken with (a, c, e) epifluorescence alone and (b, d, f) combined epifluorescence and transmitted bright field illumination. Magnifications: ×195 (a–d); ×260 (e, f).

Double staining

mAb against three T-cell surface antigens, OKT11 (pan T cells), CD3 (mature T cells), CD4 (helper T cells), one B-cell antigen (OKB1) and one IL-2 receptor (CD25) antigen, were used in this study. All double stainings were performed at the period which coincided with maximum IFN-γ production, which was 44–56 hr post-SEB-induction. Representative photomicrographs of double-staining are presented in Fig. 3. The frequency of IFN-γ-producing cells that were positive for the phenotypic markers CD3, CD4, CD25, OKT11 and OKB1 were 55%, 54%, 77%, 71% and 0%, respectively (Fig. 4a). The frequency of the various phenotypic markers on the total lymphocyte population is presented in Fig. 4b. The addition of monesin/CCCP to SEB-treated cultures did not enhance nor diminish the immunostaining of the cell-surface antigens. No membrane stainings were observed in control specimens stained with IGS alone.

IFN characterization

In order to confirm that the IFN produced by SEB-treated PBMC was indeed IFN-γ, 48–72 hr fluid samples were collected

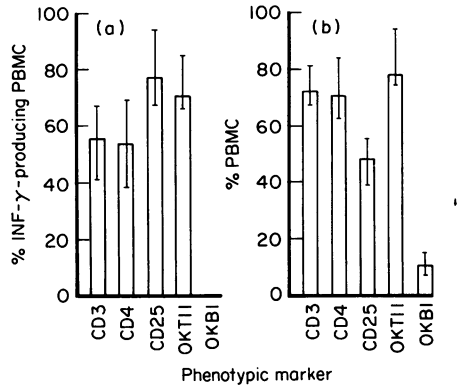

Figure 4. A comparison of the frequency (%) of IFN-γ-producing PBMC that are positive for a phenotypic marker (a), and the frequency (%) of the phenotypic marker in the total PBMC population (b). All immunostainings were performed on PBMC 44–56 hr after SEB stimulation.

in two separate experiments from two different donor PBMC. Aliquots of the fluid were incubated, respectively, with the three antibodies directed against hIFN-α, hIFN-β of hIFN-γ. IFN activity was assessed by the plaque reduction method. IFN production under the present SEB regime was approximately 420 (range 341–510) IU/ml culture fluid. No reduction in IFN titre was observed when these samples were absorbed with antibodies against hIFN-α or hIFN-β. Complete neutralization of IFN activity was demonstrated in specimens treated with anti-hIFN-γ mAb. The IFN produced by SEB induction was also abrogated by 24–48 hr pH 2-treatment.

DISCUSSION

The induction in human PBMC by SEB had been shown to be of the IFN-γ type (Svedersky *et al.*, 1982). This observation was confirmed in the present study by neutralization experiments. Furthermore, using highly specific mouse mAb against hIFN-γ, it was possible to detect cytoplasmic fluorescence in SEB-treated PBMC by indirect immunostaining procedures.

The maximum number of IFN-γ-positive cells, which made up 3·5–6% of the PBMC population, occurred 44–56 hr post-SEB stimulation. Positively stained polar bodies aligned in a ring-formation adjacent to the nuclear membrane was the most frequent fluorescent pattern observed at all stages of IFN-γ induction. Localized juxtanuclear cytoplasmic fluorescence was reported earlier in OKT3-stimulated PBMC by Laskay *et al.* (1986), who also pointed out the similarity in morphology between the IFN-γ fluorescent pattern and the Golgi apparatus. Using two different coloured conjugates, these investigators were able to show that IFN-γ was located within the parameter of the Golgi complex.

Monensin and CCCP are cationic ionophores known to interrupt subcelllar transport of a number of secretory proteins. The secretion of a large number of proteins, e.g. viral glycoproteins, proteins from fibroblasts, macrophages, procollagen as well as immunoglobulin from plasma cells can be interrupted either at the intracellular level or at the level of the Golgi complex if the cells are treated with low concentrations of these substances (Tartakoff, Hoessli & Vassalli, 1980; Tartakoff & Vassalli, 1978). We were curious as to whether monensin and CCCP could have the same effect on cytoplasmic IFN-γ and if so whether the interruption of transport, which might lead to the accumulation of this lymphokine, would result in the enhancement of IFN-γ-specific cytoplasmic fluorescence. The experimental results showed that a greater than 10- and 20-fold increase in the sensitivity of immunostaining could be obtained when IFN-induced cultures were treated with monensin and CCCP, respectively. This was true irrespective of whether the comparisons were made in cultures 24, 48 or 65 hr post-SEB stimulation. Enhanced immunostaining was observed exclusively in nuclear membrane polar body if monensin/CCCP was added to SEB-induced PBMC that were cultured for less than 24 hr or in the nuclear membrane polar body(ies) as well as in the rest of the cytoplasm if the ionophores were added to SEB-induced PBMC that were cultured for more than 24 hr. These observations suggest that the interruption of IFN-γ transport (if indeed this was the cause of the enhanced immunostaining) as a result of monensin/CCCP treatment could occur in the Golgi complex and/or in the rest of the cytoplasm. An alternate possibility, one which can not be ruled out by the present study, is that monensin/CCCP might induce changes in the cellular physiology in such a way as to increase the binding efficiency of the anti-IFN-γ –IFN-γ complex. Irrespective of the mechanisms involved, enhanced immunostaining will be useful in the demonstration of low but physiologically active levels of cytoplasmic IFN-γ. It also makes possible the use of mAb with weak binding efficiency and allows a reduction in the quantity of effective primary antibodies used; thereby substantially reducing the cost of immunofluorescence studies.

The present study demonstrated for the first time that IGS staining procedures could be used simultaneously with anti-IFN-γ mAb to detect surface antigen. Hitherto simultaneous demonstration by immunostaining of intracytoplasmic and membrane antigens has been confined to the use of two different coloured fluorescein conjugates, e.g. FITC and TRITC. While FITC presents a bright green and relatively long fluorescent half-life, the same cannot be said of TRITC or other red colour conjugates, which are less bright and are easily quenched. It is also of interest to note that the majority of the IFN-γ-producing cells in response to SEB stimulations displayed the pan T-cell surface antigen, T11 (CD2), and expressed receptors for interleukin-2. Likewise, using OKT3 as inducing agent, Andersson *et al.* (1986) have reported comparable results.

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