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Down-regulation of cytokine production and interleukin-2 receptor expression by pooled human IgG

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SUMMARY

The influence of pooled human IgG preparations for intravenous use (i.v.Ig) on *in vitro*-induced cytokine production was studied at the single-cell level using cytokine-specific monoclonal antibodies (mAb) and indirect immunofluorescent technique. Cultured mononuclear cells from peripheral blood from healthy adult donors were polyclonally stimulated for 96 hr by either direct ligation of T-cell receptors using immobilized anti-CD3 mAb or by a combination of a protein kinase C activator [phorbol 12-myristate 13-acetate (PMA)] and a calcium ionophore (ionomycin) in the absence or presence of i.v.Ig. A marked inhibition of proliferation and blast transformation was noted in all i.v.Ig exposed cultures, despite good cell survival. The production of the T-cell lymphokines interleukin-2 (IL-2), IL-10, interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β) was significantly down-regulated during the whole studied period in the i.v.Ig containing anti-CD3 stimulated cultures. The synthesis of the monokine IL-8 was not suppressed and that of TNF-α, which was made by both lymphocytes and monocytes, was only moderately inhibited. Somewhat different and more transient effects were observed in the i.v.Ig-exposed PMA/ionomycin-activated cultures. The production of IL-2, IL-3, IL-4, IL-5, IL-10, TNF-β and granulocyte–macrophage colony-stimulating factor (GM-CSF) was down-regulated during the initial phase of the cultures up to 48 hr, but not at 48–96 hr. The synthesis of IFN-γ and TNF-α was unaffected of the influence of i.v.Ig during the entire culture period. The expression of IL-2 receptors (IL-2R) was significantly suppressed in the i.v.Ig-treated anti-CD3-activated cells, but not in the PMA/ionomycin-stimulated cultures. Taken together our results indicate that pooled IgG may mediate immunomodulation by direct effects on cytokine production and on T-cell proliferation.

INTRODUCTION

Infusion of pooled human IgG in intravenous immunoglobulin preparations (i.v.Ig) has resulted in clinical improvement in a number of inflammatory diseases of presumed autoimmune aetiology (for review see refs 1,2). The mode of action of the i.v.Ig preparations in these disorders is poorly understood. We have experimentally tested the hypothesis that one mechanism could act by modulating cytokine production. We have previously reported effects by i.v.Ig on *monokine* production in cultured human blood mononuclear cells (MNC) stimulated by lipopolysaccharide (LPS) from *Escherichia coli* or by *Borrelia burgdorferi* spirochetes.[3,4] We found that interleukin-6 (IL-6) production was specifically down-regulated, while that of tumour necrosis factor-α (TNF-α), IL-1α and IL-1β was unaffected, indicating that the inhibitory effects seen on IL-6 production could not be explained by antigen neutralization. Additional experiments have shown that neither IL-8 nor IL-10 synthesis was affected by the i.v.Ig-containing cultures, while IL-1 receptor antagonist formation was increased (U. G. Andersson, L. Björck, U. Skansén-Saphir and J. P. Andersson, unpublished data). It is known from previous reports that aggregated IgG is a potent inducer of IL-1 receptor antagonist formation.[5,6] One important difference between pooled human plasma and plasma from one single donor is that up to 40% of the molecules of IgG from pooled plasma (10,000 donations) spontaneously dimerize by anti-idiotypic interactions.[7] IgG prepared from one single donor contains only trace quantities of dimers or larger immune complexes.

In this report we have focused on whether i.v.Ig can modulate *lymphokine* production in *in vitro*-activated lymphocytes. We have cultured MNC and induced direct polyclonal T-cell activation and studied the effects of i.v.Ig on the

Abbreviations: BSS, balanced salt solution; FITC, fluorescein isothiocyanate; i.v.Ig, pooled human IgG preparation for intravenous use; mAb, monoclonal antibody; MNC, mononuclear cells.

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subsequent production of IL-2, IL-3, IL-4, IL-5, IL-8, IL-10, TNF-α, TNF-β, granulocyte–macrophage colony-stimulating factor (GM-CSF) and interferon-γ (IFN-γ).

MATERIALS AND METHODS

Cell culture and cytokine induction

MNC were prepared from buffy coats of healthy adult blood donors by Lymphoprep centrifugation (Nycomed AS, Oslo, Norway) and the cells were cultured 1×10^6 cells/ml at 37° in humidified air atmosphere containing 5% CO_2, for indicated periods of time in RPMI-1640 medium (Flow Laboratories, Irvine, U.K.) supplemented with 5% heat-inactivated, LPS-free, human AB serum and 2 mM L-glutamine in pyrogene-free cell culture polystyrene wells (Costar, Cambridge, MA). The cells were either stimulated with 0·5 μM ionomycin (ATC 31005, Calbiochem, La Jolla, CA) in combination with 1 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St Louis, MO) or with immobilized anti-CD3 monoclonal antibody (mAb) (Orthoclone OKT3; Cilag AG, Schaffhausen, Switzerland). Flat-bottomed 96 microtitre wells were coated before culture with the OKT3 mAb with 100 μl/well of a 10 μg/ml solution in a coating buffer (Na_2CO_3 1·59 g, $NaHCO_3$ 2·93 g, NaN_3 200 mg, distilled water at 1000 ml, pH 9·6) for at least 4 hr at 37°. The wells were subsequently washed three times with balanced salt solution (BSS) to remove unbound antibody.

Five hundred milligrams of lyophilized i.v.Ig without preservatives (Gammagard, Baxter, Lessines, Belgium) was dissolved in 10 ml distilled H_2O. The proteins in the Gammagard preparations consist of 94% mono- or dimeric human IgG and 4% human albumin. Gammagard does not contain contaminating α_2 macroglobulin. Freshly made preparations were added at a final concentration of 6 mg/ml to the MNC 0·5 hr prior to mitogen stimulation and were kept present during the culture period. Cultures without i.v.Ig were used as controls.

Cytokine-specific antibodies

The following cytokine detecting mAb were used for indirect immunofluorescence staining: IL-2 (17.H.12), IL-3 (3.G.11), IL-4 (25.D.2) IL-5 (39.D.10), IL-10 (19.F.1 + 12.G.8), TNF-α (20.A.4), GM-CSF (21.C.11) all rat IgG mAb from J. Abrams (DNAX, Palo Alto, CA),[8] IL-8 [NAP-1 mouse IgG1 mAb from M. Ceska (Sandoz, Vienna, Austria)],[9] TNF-β [LTX-21, mouse IgG2b mAb from G. Adolf (Boehringer-Ingelheim, Vienna, Austria)][10] and IFN-γ [DIK-1 mouse IgG1 mAb from G. Andersson (KABI, Stockholm, Sweden).[11] The cytokine-specific mAb were used at a final concentration of 1–5 μg/ml.

Immunofluorescence staining of cytokines

Our method has been reported recently[12,13] and is summarized here. Cultured cells were harvested after indicated periods of time and washed in BSS (Gibco, Paisley, U.K.) supplemented with 0·01 M HEPES buffer. Cells were transferred to adhesion slides (BioRad Lab, Munich, Germany) and were allowed to adhere electrostatically to the slides for 10 min at room temperature. Excess cells were washed away and unbound surface area on the adhesive fields was blocked with 2% foetal bovine serum in BSS by a 10-min incubation. Approximately 4×10^4 cells were fixed on each field with phosphate-buffered 4% paraforaldehyde at pH 7·4 for 30 min. After subsequent washes with BSS the cells were incubated with 10 μl of the cytokine-specific mAb for 20 min in BSS supplemented with 10% human AB serum and with 0·1% saponin as a detergent, which enabled the mAb to penetrate intracellularly. The cells were washed in BSS–saponin and exposed for another 20 min to 10 μl of the fluorescein isothiocyanate (FITC)-coupled second step antibodies, which were also supplemented with 10% human AB serum. We used FITC-labelled anti-mouse IgG1 or anti-IgG2b (Caltag Laboratories, South San Francisco, CA) at a final concentration of 1:300 or FITC anti-rat IgG (Vector Laboratories, Burlingame, CA) at 1:100. After washes in BSS–saponin a final wash was performed with only BSS, which prevented leakage from the cells of stained cytokine. The cells were left to dry on the slides before they were mounted in buffered glycerol containing 2% diazobicyclo-octane to reduce ultraviolet (UV) quenching.

Surface staining and flow cytometry

We used a phycoerythrin (PE)-labelled anti-CD25 mAb (detecting the 55,000 MW subunit chain) to stain surface IL-2 receptors and a PE-labelled Leu-4 (both antibodies from Becton Dickinson, San José, CA) to visualize CD3 expression and a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) to analyse the results.

Fluorescence microscopy

The frequency of cytokine-producing cells was determined by UV microscopic evaluation of 2000 cells. The slides were examined with a Reichert-Jung fluorescence microscope equipped with a 200 W mercury lamp.

Statistical analysis

Student's *t*-test was used for all statistical analyses.

RESULTS

Cytokine production after ionomycin/PMA stimulation

We have previously reported[14] that a large number of MNC will produce lymphokines and monokines in response to ionomycin/PMA, which bypasses all normal cell surface receptors. The colour of the medium in the ionomycin/PMA-activated cultures without i.v.Ig supplementation changed from pink to yellow after 24–48 h from initiation of the cultures, as a consequence of increased cell metabolism. This shift of the colour of the cultures occurred much later and not to the same extent in the ionomycin/PMA-stimulated cell cultures, which contained i.v.Ig. The cell survival, as judged from trypan blue staining, was not reduced in the i.v.Ig-containing cultures. It was also obvious from microscopy examination that blast transformation was grossly diminished in the i.v.Ig exposed cells in comparison with the controls (Fig. 1). Cell profileration assessed by [^{3}H]thymidine incorporation was also much reduced in the i.v.Ig containing cultures (Fig. 2a).

We measured cytokine production in the cultures by studying harvested, fixed permeabilized cells by indirect immunofluorescence, using cytokine-specific mAb. Producer cells were recognized by the accumulation of the intracellular cytokines in the Golgi apparatus, which appeared with a characteristic morphology. Many cells that showed the typical local Golgi staining also expressed the cytokine in the cytoplasma and/or on the surface membrane (Fig. 1). We have previously shown that

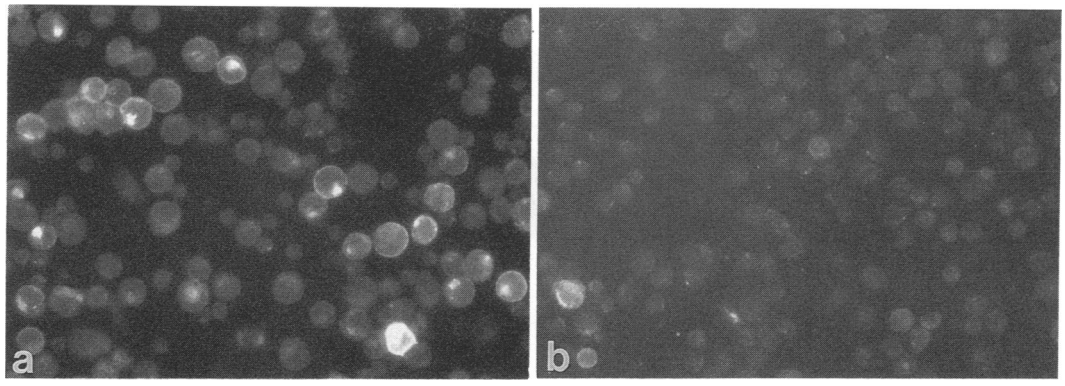

Figure 1. Photographs demonstrating TNF-β production in cultures stimulated by anti-CD3 mAb for 48 hr without (a) or with (b) i.v.Ig. Cytokine-producing cells expressed a local immunofluorescent staining representing the accumulation of the cytokine in the Golgi area with or without additional cytoplasmic and cell-surface expression. Most TNF-β-producing cells were blast transformed in the absence of i.v.Ig (a), while most cells in the i.v.Ig-exposed cultures were small and the number of TNF-β-producing cells was much reduced (b). Magnification $\times 400$.

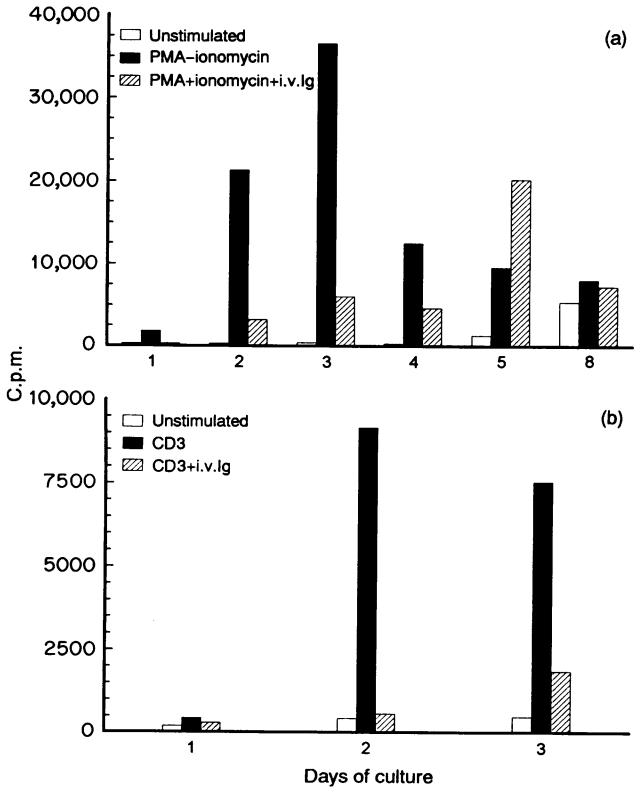

Figure 2. The influence of i.v.Ig addition on the proliferative response in the MNC cultures stimulated by PMA/ionomycin (a) or anti-CD3 mAb (b) is shown. The data represent means of triplicate assessments of [^{3}H]thymidine incorporation in one out of four separate experiments, which all gave similar results.

cells appearing with the typical Golgi staining (with or without additional cytoplasmic staining) represent cytokine *producer* cells, but not target cells, which have absorbed/endocytosed the cytokine.[12] The addition of natural or recombinant cytokines to the cultured MNC will generate a diffuse membrane or cytoplasmic staining, but never the accumulation in the Golgi organelle, the characteristic appearance by which we identify cytokine-synthesizing cells. Considerable differences in the number of cytokine-producing cells were observed between the i.v.Ig exposed and non-exposed stimulated cultures (Fig. 3). The synthesis of certain cytokines, such as IL-2, IL-3, IL-4, IL-5, IL-10, GM-CSF and TNF-β was markedly reduced up to 48 hr of stimulation in the i.v.Ig-containing cultures, while insignificant effects were seen on the capacity to produce IFN-γ and TNF-α. A shift in the kinetic response was seen later in the cultures at 72 and 96 hr for the formation of all cytokines but IL-10, when cytokine production occurred to the same or an increased extent in the ionomycin/PMA-stimulated MNC, which had been co-cultured with i.v.Ig (Fig. 3).

We also analysed whether the addition of i.v.Ig to the staining step with anti-cytokine mAb would influence the observed results. We did not see any change in the number of stained cells or the intensity of the stainings. These findings did thus not support the existence of cytokine neutralizing antibodies in the i.v.Ig preparations as the explanation for down-regulation of cytokine synthesis.

Cytokine production and cell-surface antigen expression in the anti-CD3-stimulated MNC

The ligation of the physiological T-cell activation receptor CD3 with the solid phase bound anti-CD3 mAb also resulted in polyclonal T-cell activation[15] with strong lymphokine production (Fig. 4). The addition of i.v.Ig from start of these cultures led to a much retarded shift of the colour of the culture medium, reduced labelled thymidine incorporation in a proliferation assay (Fig. 2b) as well as a strong inhibition of blast transformation. These findings thus parallel those in the ionomycin/PMA-activated cultures. A contrasting result was the down-regulation of the IL-2 receptors (CD25) in the i.v.Ig-exposed anti-CD3-stimulated cultures, but not in the PMA/ionomycin-activated cells (Table 1, Fig. 5). The effects of i.v.Ig on cytokine production also differed in the anti-CD3-stimulated cells versus those found in the ionomycin-PMA cultures. The pooled human IgG preparation exerted a significant inhibition in the anti-CD3 mAb-stimulated cultures in the production of four of the studied lymphokines, that is IL-2, IL-10, IFN-γ and TNF-β ($P < 0{\cdot}01$). The suppressive effects of the i.v.Ig supplementation

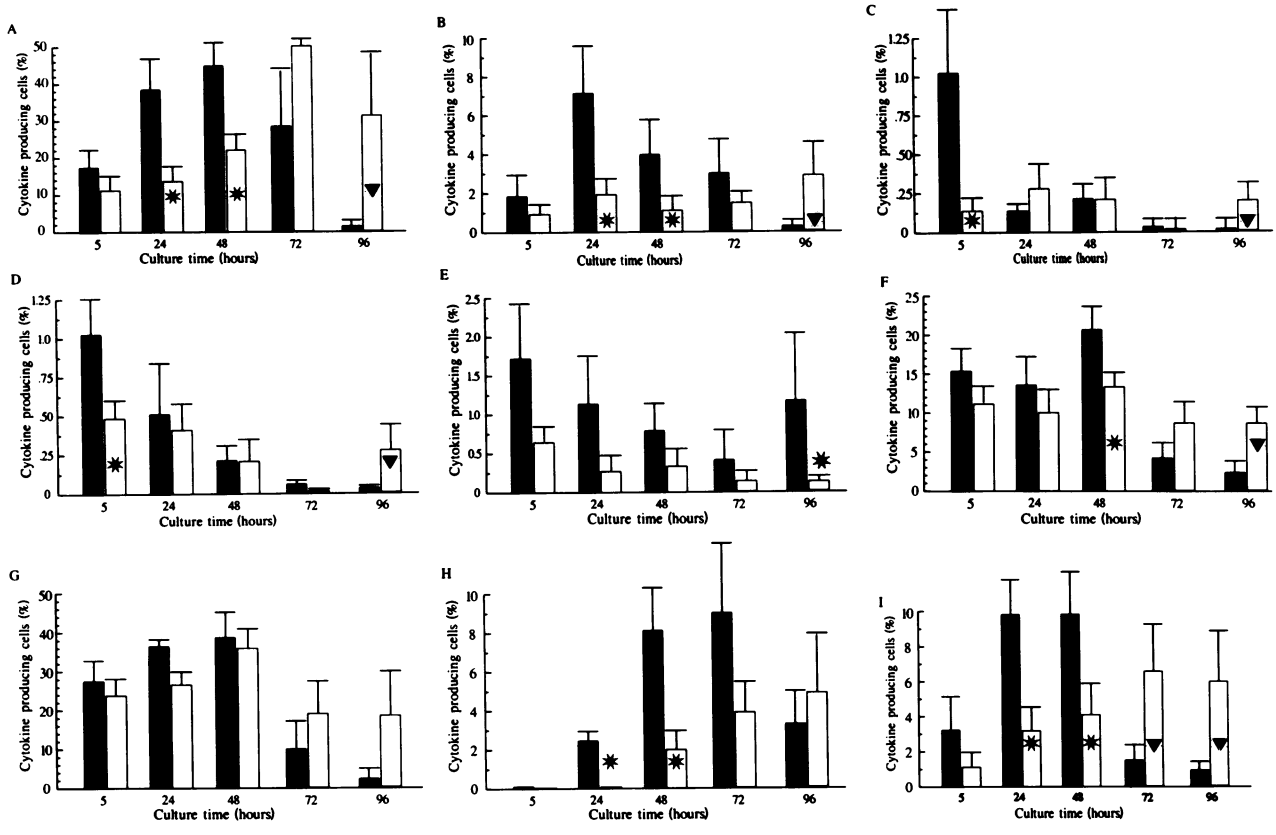

Figure 3. The frequency and kinetics of cytokine-producing cells after PMA/ionomycin stimulation is demonstrated in the presence (□) or absence (■) of i.v.Ig. The following cytokines were studied: IL-2 (A); IL-3 (B); IL-4 (C); IL-5 (D); IL-10 (E); IFN-γ (F); TNF-α (G); TNF-β (H); GM-CSF (I). The results represent means ± SD of seven different experiments using seven different donors. *Significant ($P < 0.05$) reduction in i.v.Ig-exposed cultures compared to controls; ▼ significantly ($P < 0.05$) increased numbers of producer cells in the i.v.Ig-supplemented cultures.

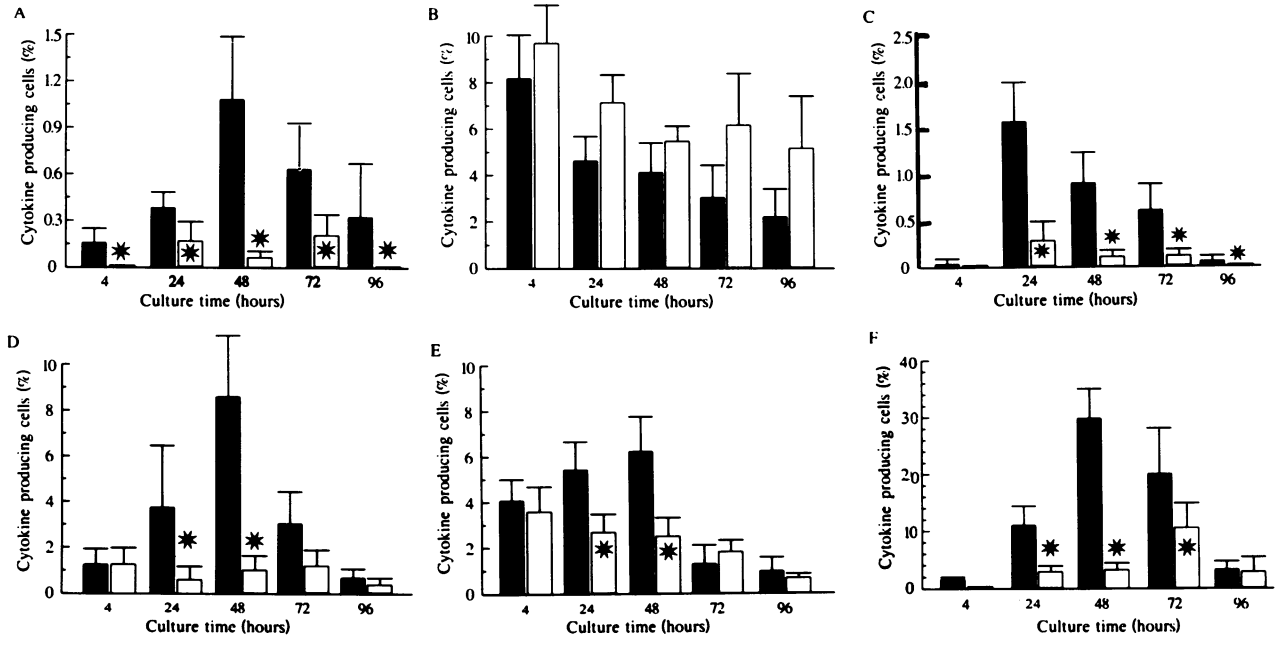

Figure 4. The percentage of cytokine-producing cells after immobilized anti-CD3 stimulation with (□) or without (■) i.v.Ig supplementation. The following cytokines were studied: IL-2 (A); IL-8 (B); IL-10 (C); IFN-γ (D); TNF-α (E); TNF-β (F). Data represent means ± SD of seven different experiments. *Significant ($P < 0.05$) reduction in i.v.Ig-exposed cultures compared to controls.

Table 1. T-cell receptor (CD3) and IL-2 receptor (CD25) expression on cells cultured with or without pooled human IgG (i.v.Ig)

		Percentage positive cells†		
		Culture time (hr)		
Phenotype	Mode of activation	24	48	72
CD3	Unstimulated	64±7‡	63±13	68±12
CD3	Anti-CD3 mAb	48±15	50±10	64±10
CD3	Anti-CD3 mAb+i.v.Ig	67±11*	61±13	65±8
CD3	PMA-ionomycin	28±11	16±10	18±16
CD3	PMA-ionomycin±i.v.Ig	44±14	25±14	23±15
CD25	Unstimulated	3±1	4±3	4±4
CD25	Anti-CD3 mAb	26±12	44±11	52±13
CD25	Anti-CD3 mAb+i.v.Ig	9±7*	16±10*	16±11*
CD25	PMA-ionomycin	22±10	46±14	50±17
CD25	PMA-ionomycin+i.v.Ig	16±6	33±11	39±11

† The percentage of positive cells as assessed by flow cytometry.

‡ Data represent means±SE of five separate experiments.

* Indicates a statistically significant difference ($P<0.05$) in surface CD expression in mitogen-activated MNC with or without i.v.Ig addition.

remained during the 96 studied hr, in contrast to the results in the ionomycin-PMA-activated cultures. No inhibitory effects were observed on the synthesis of the monokine IL-8 and only a moderate reduction of TNF-α, which was produced by lymphocytes as well as monocytes.

DISCUSSION

We believe that this is the first report of i.v.Ig down-regulation of T-cell blast transformation, expression of IL-2R, proliferation of T cells and the production of certain T-cell lymphokines. These effects of i.v.Ig were actually more impressive than those that we and others[3,16-18] have reported concerning i.v.Ig influence on monokine synthesis. It is likely that we actually underestimated the suppressive effects exerted by the i.v.Ig preparations on the production of the lymphokines, by the fact that our method is semi-quantitative in assessing only numbers of cytokine-producing cells. It was quite obvious from microscopic examination that the big blast transformed cells in the non-i.v.Ig-exposed activated cultures contained much more cytokine than the smaller i.v.Ig-treated cells with their correspondingly smaller Golgi organelles (Fig. 1).

Our results indicate an effect of the i.v.Ig preparations on the *production* of cytokines rather than on the *uptake* of cytokines by target cells, since we have only assessed cells with the typical accumulation of the cytokines in the Golgi organelle. We have shown in previous studies that this characteristic morphology of the staining is generated by active synthesis, but never by adsorption or endocytosis of cytokines.[12]

We do not know whether these effects were mediated by the Fab or Fc part of the IgG molecules, or whether the effects were exerted directly on the T cells or via accessory cells. Future experiments with fragmented i.v.Ig preparations and purified subsets of MNC or T-cell clones will be required to settle these questions. We have no indication that cytotoxic effects of the

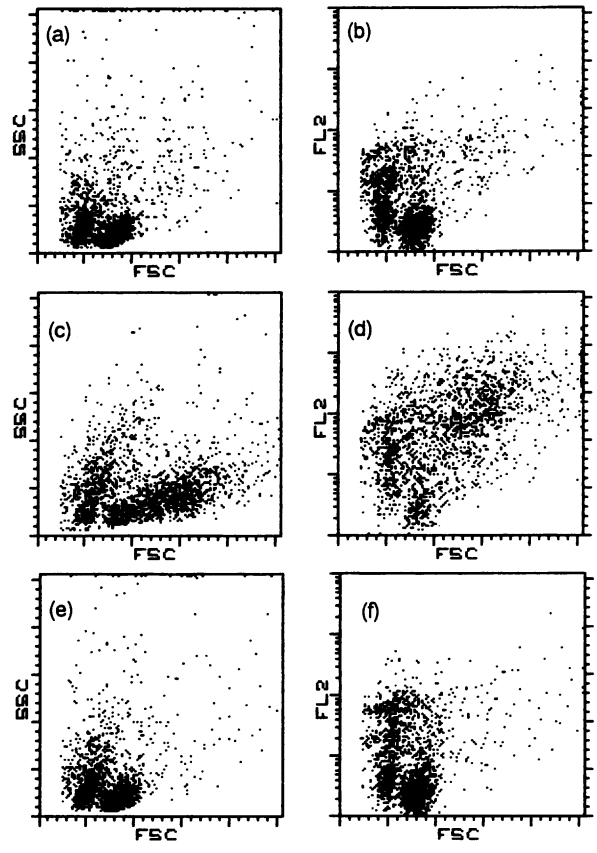

Figure 5. Flow cytometry analysis of cell size and surface expression of IL-2R (CD25) in cells cultured for 72 hr without any stimulation (a, b), or with solid phase bound anti-CD3 mAb (c, d) or with anti-CD3 mAb and i.v.Ig (e, f). The i.v.Ig-exposed anti-CD3-stimulated cells were similar in size and complexity to the unstimulated cells and the IL-2R expression was markedly reduced. Totally ungated data are shown and the aquisition was made by logarithmic mode. One of five separate experiments with similar results is shown.

cells are the mechanism for i.v.Ig suppression, since cell survival was similar in the i.v.Ig-containing cultures and the controls. The fact that the production of certain studied cytokines was not or very little affected is another argument against cytotoxicity, unspecific toxic effects of direct neutralization of the mitogen as the mode of effector mechanism. We have no evidence for neutralizing cytokine-specific antibodies in the i.v.Ig preparations either, since the addition of i.v.Ig to the staining procedure did not influence the subsequent cytokine staining. We did not observe induction or overproduction of any studied inhibitory cytokine as the cause of the i.v.Ig-mediated suppression. Although we did not assess the formation of IL-1 receptor antagonist, we find it unlikely that this cytokine would prevent ionomycin/PMA activation of T cells, which is a monocyte-independent way of cell stimulation. The inhibitory effects of i.v.Ig can thus probably not be explained by a suppression of co-stimulatory signals delivered by monocytes, but rather indicate a direct action on the T cells. We also found that IL-2 receptor expression was reduced in the anti-CD3 mAb-treated cells, but not in the PMA-ionomycin-stimulated cells—yet proliferation was significantly reduced by i.v.Ig in both culture systems. Either the reason for this inhibition differs between the two modes of stimulation, or the down-regulation of IL-2R was not

the primary cause of this reduction. The reduced IL-2R expression would then rather be the consequence of an interference by i.v.Ig in the early stages of T-cell activation, also reflected by the lack of blast transformation in the i.v.Ig exposed cultures (Figs. 1 and 5).

The dose of 6 mg/ml i.v.Ig that we have chosen to study experimentally corresponds to what can be obtained therapeutically *in vivo*. It has been reported that an i.v.Ig infusion of 0·5 g/kg body weight results in an average IgG serum increment of 10 mg/ml.[19] The critical question whether our observations on i.v. Ig modulation of *in vitro* lymphokine production has any bearing on the therapeutical effects seen *in vivo* in certain autoimmune diseases will require further study. We wish to focus interest on this intriguing possibility. To understand the mode of action of i.v.Ig in various autoimmune diseases would help us to clarify important pathogenic mechanisms in these disorders.

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