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Inhibition of CD25 (IL-2Rα) expression and T-cell proliferation by polyclonal anti-thymocyte globulins

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SUMMARY

Anti-lymphocyte and anti-thymocyte globulins (ATG) are currently used as immunosuppressive agents in organ transplantation. Their administration *in vivo* may induce not only lymphocyte depletion but also functional effects which were investigated in the present study. *In vitro* ATG inhibited T-cell proliferation induced by monocyte-dependent T-cell mitogens, like CD3 antibodies, phytohaemagglutinin (PHA) and concanavalin A (Con A), by monocyte-independent mitogens, like CD2 antibodies, or by protein kinase C activators (phorbol esters) associated with a calcium ionophore. The inhibitory effect of ATG was therefore not solely accounted for by a suppression of co-stimulatory signals delivered by monocytes, but rather implied a direct action on T cells. Addition of recombinant human interleukin-2 (rIL-2) did not overcome the inhibition. Suppression of T-cell proliferation by ATG was characterized by normal RNA synthesis and IL-2 secretion contrasting with markedly reduced expression of the CD25 protein [p55, the α-chain of interleukin-2 receptor (IL-2R)] both in cytoplasm and on T-cell membrane, as well as a decreased secretion of interferon-γ (IFN-γ). Northern blot analysis revealed increased levels of CD25 and IFN-γ mRNA, suggesting a post-transcriptional inhibition of these molecules, whereas IL-2 mRNA levels were unchanged. These data demonstrate that inhibition of T-cell proliferation by ATG can be attributed primarily to a post-transcriptional defect of CD25 expression, implying a novel mechanism different from those described with other immunosuppressive agents. Blocking of T-cell proliferation in the late G1 phase of the cell cycle may contribute to the immunosuppressive activity of ATG in prophylactic treatment of allograft rejection.

INTRODUCTION

Activation of resting T lymphocytes can be induced by a variety of ligands, e.g. lectins or monoclonal antibodies (mAb) directed against cell membrane molecules, in presence or absence of accessory cells, or alternatively by direct stimulation of intracellular signals by a combination of calcium ionophores and protein kinase C activators.[1,2] T-cell mitogens trigger a coordinated sequence of subsequent events including the expression of early activation genes (e.g. *c-fos* and *c-myc*) followed by progression to the G1 phase of the cell cycle, activation of cytokine-gene transcription and subsequent cytokine synthesis. Progression into S/G2 phase requires the binding of interleukin-2 (IL-2) to the high-affinity heterodimeric interleukin-2 receptor (IL-2R).[3,4] Immunosuppressive agents such as cyclosporin A (CsA), FK506 and rapamycin (RPM) act at discrete stages of T-cell activation.[5] CsA and FK506 are potent inhibitors of transcription of early T-cell activation genes and block the progression into the G1 phase of the cell cycle. They inhibit the binding of nuclear factors to IL-2 enhancer sequences and subsequent transcription of IL-2 gene.[6] Conversely RPM was reported to block a later event of T-cell proliferation by inhibiting intracellular signals generated by the binding of IL-2 to IL-2R.[7]

Anti-lymphocyte or anti-thymocyte globulins (ATG) prepared by immunizing horses or rabbits with human lymphocytes, thymocytes, T or B lymphoblasts, have been used as immunosuppressive agents for more than 20 years. Numerous studies have demonstrated their efficacy both in prophylaxis and in treatment of established rejection.[8] We have previously reported that ATG contain antibodies directed against func-

Abbreviations: ATG, anti-thymocyte globulins; BSA, bovine serum albumin; Con A, concanavalin A; CsA, cyclosporin A; FCS, foetal calf serum; FITC, fluorescein isothiocyanate conjugated; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PHA, phytohaemagglutinin; PMA, phorbol myristate acetate; PPD, purified protein derivative; RPM, rapamycin; SEB, staphyloccocal enterotoxin B; TTG, antitetanus toxoid globulins.

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tional leucocyte molecules, such as CD2, CD3, CD4, HLA DR and LFA-1.[9] Therefore, in addition to lymphocyte depletion observed *in vivo*, ATG may also modulate T-cell responses. Early studies have shown that ATG themselves can induce T-cell proliferation but they may also inhibit T-cell proliferation induced by other agents.[10,11] Inhibition of mixed lymphocyte reaction and T-cell responses to tuberculin purified protein derivative (PPD) are observed at infra-stimulatory concentrations of ATG, whereas higher concentrations are required to suppress phytohaemagglutinin (PHA)-induced T-cell proliferation. In a recent report we have demonstrated an anti-proliferative effect of ATG on B cells and B-cell lines at concentrations where ATG induce T-cell proliferation.[12]

In this study we analysed the inhibitory properties of ATG on T-cell proliferation. We report that the inhibitory effect of ATG results from a direct interaction of ATG with T cells, and does not depend on monocytes. ATG inhibited CD25 expression and interferon-γ (IFN-γ) secretion, whereas IL-2 secretion in cell culture supernatant was not decreased. An increase of CD25 and IFN-γ mRNA expression without intracytoplasmic accumulation of CD25 molecules suggests that ATG prevents progression of activated T cells to the S phase by inhibiting CD25 expression at a post-transcriptional level.

MATERIALS AND METHODS

Anti-thymocyte globulins

Five ATG batches were provided by Pasteur Merieux Serums & Vaccins (Lyon, France). Their main characteristics have been described in a previous report.[9] These ATG batches include horse anti-human B lymphoblastoid cell lines (nos. 11 and 12), horse (no. 9) and rabbit (no. 4) anti-human thymocytes, and horse anti-thoracic duct lymphocytes (no. 7). Horse antitetanus globulins (TTG) and normal rabbit IgG were used as controls.

Cell preparation

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by centrifugation of heparinized blood on a layer of Ficoll Hypaque (Lymphoprep, Nycomed, Oslo, Norway). Cells were washed three times in Hanks' balanced salt solution before culture. Peripheral blood lymphocytes (PBL) were prepared from PBMC by elimination of monocytes and natural killer cells by L-leucine methyl ester treatment according to Thiele *et al.*[13] L-leucine methyl ester is a lysomotropic agent rapidly metabolized into leucine inside the lysosomes, causing osmotic swelling of these organelles and subsequent cell death. Monocyte depletion was assessed by flow cytometric analysis after staining with Leu-M3 (CD14) mAB (Becton Dickinson, Pont de claix, France).

Cell culture

Cells were resuspended in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) (Seromed, Biokrom K. G, Berlin, Germany), 2 mM L-glutamine and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). Cells (1×10^6/ml) were incubated in 96-well microplates (Costar, Cambridge, MA) in the presence of mitogen. The mAb OKT3 (Ortho Pharmaceutical Corporation, Raritan, NJ) was used at the final concentration of 100 ng/ml. Monoclonal antibodies D66 plus X11 (CD2 mAb) were a generous gift from Professor A. Bernard (Nice, France) and each ascites fluid was used at 1:400 for optimal mitogenic activity on human T cells. Phytohaemagglutinin (Wellcome, Dartford, U.K.) was used at 10 μg/ml. The staphyloccocal enterotoxin B (SEB), concanavalin A (Con A), phorbol myristate acetate (PMA) and ionomycin, obtained from Sigma Chemicals (St Louis, MO), were used at 1μg/ml, 2·5 μg/ml, 10 ng/ml and 1 μg/ml respectively. ATG were added at the beginning of the culture at the indicated concentration. Cultures were maintained in a humidified atmosphere containing 5% CO_2. For measurement of DNA synthesis, cells were pulsed with 1 μCi/well of [^{3}H]TdR (CEA, Saclay, France) during the last 12 hr of a 3-day culture period. For RNA synthesis cells were pulsed with 1 μCi/well of [^{3}H] UdR (CEA) during the last 6 hr of a 24-hr culture period. [^{3}H]TdR and [^{3}H]UdR incorporations were measured using a Packard scintillation counter by standard liquid scintillation counting after harvesting.

Cytokine assays

Cell-free supernatants were harvested after 48 hr of culture and concentrations of immunoreactive IL-2 and IFN-γ were determined using a sandwich-type ELISA method. Monoclonal antibodies BG5 and BG1, respectively anti-IL-2 and anti-IFN-γ were obtained from J. Wijdenes (CRTS Besançon, France). They are both IgG1 purified from ascites fluid and they exhibit specific neutralizing activity towards human IL-2 and IFN-γ, respectively. They were diluted at 2 μg/ml in veronal-buffered saline (VBS) pH 8·6 for coating microplates (Costar). After three washes in VBS containing 0·1% Tween 20, plates were saturated with 200 μl of VBS containing 1% bovine serum albumin (BSA) and 0·1% Tween 20 for 1 hr. Then, 100 μl of serial dilution of culture supernatant was added to duplicate wells. After 1 hr at 37° and three washes, 100 μl of goat anti-human IL-2 or goat anti-human IFN-γ serum (obtained from J. Wijdenes) were added to appropriate wells. After incubation and three washes, 100 μl of rabbit radish peroxidase-conjugated anti-goat IgG (Jackson, Bar Harbor, ME) was added and incubated for 1 hr at 37°. After five washes, ortho phenyl diamine (Sigma) was added to each well. Microplates were incubated for 15 min at room temperature and the reaction was stopped by the addition of 100 μl of H_2SO_4 (2N) to each well. Recombinant human IFN-γ (kindly provided by Professor Weissmann, Zurich, Switzerland) and recombinant human IL-2 (Cetus Corporation, 3×10^6 U/ml) were used for calibration.

Immunofluorescence assays

For analysis of membrane IL-2R expression, cells ($0{\cdot}5 \times 10^6$) were incubated with fluorescein isothiocyanate (FITC) anti-IL-2R mAb (Becton Dickinson) for 30 min at 4°. After two washes in isotonic NaCl/Pi buffer containing 1% BSA and 0·2% NaN_3 (PBS/BSA/azide), cells were fixed with 1% paraformaldehyde in PBS/BSA/azide.

For intracellular analysis of IL-2R expression, cells were permeabilized by treatment with saponin (Sigma). A pellet of 5×10^5 cells was resuspended in 100 μl of 0·33% saponin in phosphate-buffered saline (PBS) containing 1% BSA (PBS/BSA/saponin) and incubated for 15 min at 4°. After two washes in PBS 1% BSA, cells were incubated with FITC anti-IL-2R mAb (Becton Dickinson) diluted in PBS/BSA/saponin, for 30 min at 4°. After two washes cells were immediately analysed on FACStar cytofluorimeter (Becton Dickinson).

RNA isolation and Northern blot analysis

Total cellular RNA was isolated from PBMC by the guanidinium-CsCL procedure and quantified by absorbance at 260 nm. RNA samples (12 μg) were denaturated, subjected to electrophoresis in 1% agarose gels containing 6% formaldehyde, and transferred to nitrocellulose membranes (Schleicher & Schuell, Ecquevilly, France). RNA was fixed to membranes by baking for 2 hr at 80°. Membranes were treated at 42° for 24 hr with prehybridization solution (50% formamide, 100 μg/ml denatured salmon sperm DNA, 5 × Denhardt's solution, 0·25% SDS, 1% glycine) and were hybridized at 42° for 48 hr in the same solution in presence of specific ^{32}P cDNA probe radiolabelled by nick translation. After washing twice for 30 min at 60° in 0·1% SSC (15 mM NaCl, 1·5 mM sodium citrate) and 0·1% SDS, membranes were exposed at −70° to X-ray film (Amersham, Paris, France). The quantity and the quality of the total RNA applied to each line were checked by methylene blue coloration of the nitrocellulose membrane (data not shown). Films were analysed with a Quantimet 570 (LEICA, Lyon, France) for quantification of radioactive signal.

cDNA probes

All the probes were human cDNA. The 0·134 kilobase (kb) IFN-γ insert in pGEM-3 was obtained from C. Martens (DNAX, Palo Alto, CA). The 0·35 kb IL-2 insert in pGEM-3 and the 1·16 kb IL-2Rα insert in pGEM-3 were respectively obtained from Z. Wang and R. de Waal Malefyt (Schering Plough, Dardilly, France). Plasmid DNA were linearized with the appropriate restriction enzyme and purified by agarose gel electrophoresis before nick-translation and labelling.

RESULTS

Inhibition of T-cell stimulation induced by accessory cell-dependent and -independent pathways

All ATG batches induced a strong T-cell proliferation with maximal DNA synthesis at 0·25–0·50 mg/ml of ATG peaking at 72 hr and rapidly decreasing thereafter. At higher concentrations, ATG did not stimulate DNA synthesis (Fig. 1). Next PBMC were stimulated by mitogenic lectins PHA or Con A, mAb OKT3, a combination of CD2 mAb D66+X11 or a combination of phorbol ester (PMA) plus calcium ionophore (ionomycin). Addition of ATG at a concentration of 1·25 mg/ml at the start of culture inhibited T-cell proliferation stimulated by the above-mentioned agents (Table 1). Inhibition was observed with five different batches obtained by immunization of horses (ATG no. 7, no. 9, no. 11 and no. 12) or rabbits (ATG no. 4) with different sources of antigen (Table 2). No significant inhibition was observed in the presence of normal rabbit IgG or horse antitetanus globulins at the same concentration as ATG. ATG did not exert their inhibitory effect by blocking the interaction of mitogenic stimuli with the cells, because preincubation of PBMC with mitogenic stimuli for 30 min to 20 hr did not abrogate the inhibitory activity of ATG. (data not shown).

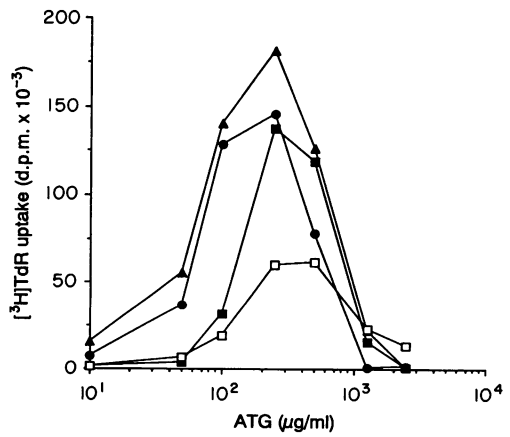

Figure 1. Mitogenic effect of ATG on PBMC of normal donor. Cells were incubated in presence of ATG no. 4 (●), ATG no. 7 (■), ATG no. 9 (▲) and ATG no. 11 (□), [³H]TdR uptake was measured during the last 12 hr of a 3-day culture period. Results are triplicates from a single experiment representative of three others.

Table 1. Effect of addition of ATG on T-cell proliferation.*

	Control	ATG†
Exp. 1		
Medium	0·68 ± 0·01‡	22·86 ± 0·99
PHA	77·91 ± 13·24	20·39 ± 1·29
Con A	72·72 ± 6·02	22·29 ± 2·16
OKT3	39·52 ± 0·08	20·97 ± 0·57
PMA + ionomycin	75·80 ± 6·28	17·72 ± 0·43
Exp. 2		
Medium	0·71 ± 0·13	22·08 ± 1·87
OKT3	98·84 ± 5·26	10·94 ± 1·95
Anti-CD2	74·20 ± 4·54	22.62 ± 2·48
PMA + ionomycin	42·94 ± 11·3	3·48 ± 0·13

* PMBC were cultured in the presence of PHA (10 μg/ml), Con A (2·5 μg/ml), OKT3 (100 ng/ml), PMA (50 ng/ml) plus ionomycin (1 μg/ml), or the association of anti-CD2 mAb (D66 plus X11) ascites (1:400).

† ATG were added at 1·25 mg/ml at the initiation of the culture.

‡ Results are expressed as d.p.m. × 10^{-3} (mean ± standard deviation).

Table 2. Inhibition of T-cell proliferation by different batches of ATG

	ATG (1·25 mg/ml)				
Mitogen†	no. 4	no. 7	no. 9	no. 11	no. 12
PHA	73*	96	ND‡	ND	ND
Con A	69	56	69	58	56
OKT3	49	48	52	56	63
Anti-CD2	ND	45	52	48	57
PMA + ionomycin	77	93	95	71	82

† PBMC were cultured in presence of (10 μg/ml), Con A (2·5 μg/ml), OKT3 (100 ng/ml), PMA (50 ng/ml) plus ionomycin (1 μg/ml) or the association of anti-CD2 mAb (D66 plus X11) ascites (1:400).

* Results are expressed as per cent of inhibition with reference to control TTG (nos. 7, 9, 11 and 12) or normal rabbit IgG (no. 4) at 1·25 mg/ml.

‡ ND, not determined.

Table 3. ATG-induced inhibition in the absence of monocytes

	PBMC	PBL*
Medium	0·32±0·03†	0·32±0·07
OKT3	26·92±2·90	0·36±0·04
PMA+ionomycin	32·62±2·87	49·62±0·99
ATG	13·92±0·59	8·43±0·18
PMA+ionomycin+ATG	1·33±0·12	0·81±0·02

Cells were cultured in the presence of OKT3 (100 ng/ml), PMA (50 ng/ml) plus ionomycin (1 μg/ml). ATG were used at 1·25 mg/ml.

* PBL were obtained by treatment of PBMC according to the Thiele method.[13]

† Results are expressed as d.p.m. $\times 10^{-3}$ (mean ± standard deviation).

At 0·25–0·50 mg/ml of ATG the level of DNA synthesis was similar, whether additional mitogen was present or not. Therefore the mitogenic activity of ATG precluded the study of their possible blocking effect at concentrations below 1 mg/ml.

Our data indicated that at high concentrations (> 1 mg/ml) ATG inhibited T-cell activation induced either by accessory cell-dependent or -independent pathways, suggesting a direct effect on T cells rather than an interference with signals delivered by accessory cells. In order to confirm this hypothesis, similar experiments were performed on cell suspensions depleted for monocytes by L-leucine methyl ester treatment. In agreement with previous reports,[14] T-cell proliferation induced by the mAb OKT3 was abrogated in such a system. Conversely T-cell proliferation induced by PMA plus ionomycin was either not modified or enhanced after treatment with L-leucine methyl ester, but the addition of ATG markedly reduced T-cell proliferation (Table 3). From these results we concluded that ATG acted directly on T cells and that their inhibitory effect was not restricted to suppression of co-mitogenic signals generated by monocytes.

Since low concentrations of ATG (1–10 μg/ml) were previously reported to inhibit mixed lymphocyte reactions and T-cell proliferative responses to PPD antigen,[10,11] we tested the effect of low concentrations of ATG on T-cell stimulation induced by CD2 or CD3 mAb, lectins (PHA and Con A), the SEB superantigen and the combination of PMA plus ionomycin. Our results indicated that only OKT3 mAb-induced proliferation was inhibited in the presence of 10 μg/ml of ATG, whereas responses to other mitogens were not affected (data not shown).

Inhibition of CD25 membrane expression and IFN-γ synthesis without impairment of IL-2 secretion

To characterize further the mechanism by which ATG affected T-cell proliferation, we studied the effect of ATG addition on sequential steps in T-cell activation including RNA synthesis measured during the first 24 hr of culture, CD25 membrane expression and lymphokine secretion.

As shown in Table 4, the presence of 1·25 mg/ml of ATG during the 3-day culture period, markedly reduced DNA synthesis in PBMC stimulated by the association of PMA plus ionomycin (90% inhibition) whereas RNA synthesis was not significantly affected (never exceeding 30% inhibition in three separate experiments). Addition of recombinant human IL-2, at doses which induced maximal proliferation of IL-2-dependent CTLL-2 cell line (25 IU/ml) in our experiments, did not prevent ATG-induced inhibition (Table 5), suggesting that inhibition of T-cell proliferation induced by ATG did not result from a defect of IL-2 production.

These results were confirmed by measurement of IL-2 secretion in cell culture supernatants. Lymphokine secretion was measured by IFN-γ and IL-2-specific ELISA. ATG addition reduced IFN-γ secretion (72% inhibition) whereas IL-2 secretion was enhanced (Table 4). ATG addition almost reduced CD25 expression to background level as determined by surface immunofluorescence analysis. These data confirmed that inhibition induced by ATG did not result from a defect of IL-2 production, but rather from a lack of CD25 expression. The lack of inhibition of RNA synthesis as well as the increase of IL-2 production at 24 hr argue against a direct effect of ATG on T cells.

Effect of ATG on IFN-γ, IL-2 and IL-2Rα (CD25) mRNA gene expression

Addition of ATG to PBMC stimulated by PMA plus ionomycin inhibited CD25 expression, IFN-γ production but not IL-2 production. To determine the level at which CD25 expression and IFN-γ production were blocked, RNA was isolated from PBMC that had been stimulated for 14 hr with PMA plus ionomycin in presence or absence of ATG. RNA was hybridized

Table 4. Effect of ATG on nucleic acid synthesis, IFN-γ, IL-2 secretion and CD25 expression in PBMC cultures

	DNA*	RNA*	IFN-γ†	IL-2†	CD25‡
Medium	0·97±0·22	2·24±0·30	0	0	7·1
PMA+ionomycin	29·81±1·69	13·85±1·13	19·5±0·3	20·6±3·1	56·4
ATG	8·11±2·71	10·26±0·63	0·5±0·03	0·6±0·1	20·6
PMA+ionomycin+ATG	2·91±0·43	11·45±1·13	5·6±0·1	26·3±1·1	11·9

* [^{3}H]TdR and [^{3}H]UdR incorporation was measured during the last 12 hr of a 3-day culture period and the last 6 hr of a 24-hr culture period. Results are expressed as d.p.m. $\times 10^{-3}$ (mean ± standard deviation).

† IFN-γ and IL-2 secretion were determined by ELISA as described in Materials and Methods, and results are expressed in IU/ml.

‡ CD25 membrane expression was determined by immunofluorescence assay, results are expressed as per cent of labelled cells.

with cDNA derived probes specific for human CD25, IFN-γ and IL-2.

As shown in Fig. 2, an increased level of mRNA for CD25 and IFN-γ was found in the presence of ATG, suggesting that inhibition of CD25 expression and IFN-γ production by ATG occurs at a post-transcriptional level. Conversely levels of mRNA for IL-2 were not altered by addition of ATG to cell cultures. As mRNA quantity was controlled to be the same (12 μg) in each lane, the IL-2 mRNA level could therefore be used as an internal control for estimating the magnitude of increase of IFN-γ and CD25 messages. In view of unaltered levels of IL-2 mRNA, it is possible that the increase of IL-2 concentration in cell culture could reflect a defective consumption of the molecule as a consequence of inhibited expression of IL-2R α-chain and subsequent diminution of the number of high-affinity heterodimeric IL-2 receptors.

Table 5. ATG-induced inhibition of T-cell DNA synthesis in presence of rIL-2

rIL-2*	0	25 IU/ml
Medium	1·66 ± 0·53†	ND
PMA + ionomycin	52·38 ± 0·13	53·25 ± 2·97
ATG	3·11 ± 0·13	2·94 ± 0·43
PMA + ionomycin + ATG	5·53 ± 0·29	5·46 ± 0·34

* PBMC were cultured in presence of PMA (50 ng/ml) plus ionomycin (1 μg/ml) and ATG (1·25 mg/ml). Recombinant IL-2 was added at the beginning of the culture.

† [^{3}H]TdR incorporation was measured during the last 12 hr of a 3-day culture period. Results are expressed as d.p.m. × 10^{-3} (mean ± standard deviation).

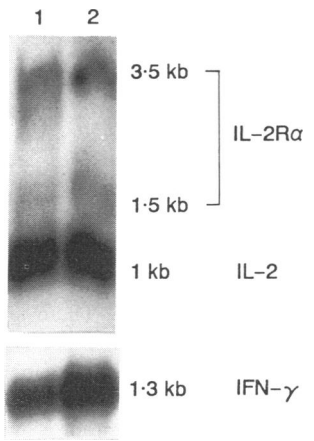

Figure 2. Effect of ATG on IL-2Rα, IL-2 and IFN-γ mRNA expression in PBMC activated by PMA plus ionomycin. After 14 hr of culture cells were lyzed, total RNA was prepared and Northern blot analysis performed. The membrane was hybridized with probes for IL-2Rα, IL-2 and IFN-γ. Lane 1, PMA (50 ng/ml) + ionomycin (1 μg/ml); lane 2, PMA (50 ng/ml) + ionomycin (1 μg/ml) + ATG no. 9 (1·25 mg/ml). Quantitative analysis of films by quantimetry demonstrated increased of IFN-γ and IL-2Rα mRNA in lane 2, whereas IL-2 mRNA levels were not significantly different.

Absence of intracytoplasmic accumulation of CD25 in ATG-treated cells

Because of increased CD25 mRNA level contrasting with defective CD25 membrane expression, we performed intracellular immunofluorescence staining in search of a possible intracytoplasmic accumulation of CD25 molecule. PBMC stimulated for 48 hr with PMA plus ionomycin in presence or absence of ATG, were stained with anti-IL-2R FITC mAb to detect CD25 expression at cell surface. In parallel cells were treated with saponin before staining with anti-IL-2R FITC mAb, to detect intracellular CD25. As reported in Fig. 3 and confirming data presented in Table 4, ATG addition markedly reduced membrance expression of CD25 in T cells stimulated by PMA plus ionomycin. The reduced level of CD25 membrance expression was accompanied by a decrease of intracytoplasmic CD25. These data suggested that the accumulation of CD25 mRNA, observed in the presence of inhibitory concentration of ATG resulted from a defect of mRNA translation, rather than a defect of CD25 export from the cytoplasm to the membrane.

DISCUSSION

In this study we investigated the effect of ATG on T-cell activation induced by different stimuli including direct activators of intracellular signal transduction pathways. The inhibitory effect described in this study was observed at supramitogenic concentrations of ATG (> 1 mg/ml) but not at low concentrations (1–10 μg/ml) except for OKT3-induced stimulation. Inhibition was observed with five different batches of ATG, irrespective of their mitogenic strength, their origin (horse or rabbit) or the source of antigen (thymocytes, thoracic duct cells, B lymphoblasts) used for immunization. It could be attributed to antibodies directed against surface molecules of lymphocytes, because control horse and rabbit Ig preparations had no effect on T-cell proliferation.

The inhibition was not due to a steric hindrance of the mitogen binding to membrane receptors by ATG because: (1) preincubation of PBMC with mitogen did not reduce the inhibitory effect of ATG, and (2) ATG inhibited stimulation induced by PMA plus ionomycin which act directly at the intracellular level.

The inhibitory effect of ATG was demonstrable in monocyte-independent activation models using CD2 antibodies,[15] or PMA plus ionomycin,[16] suggesting that ATG did not merely suppress cognate interaction or co-stimulatory signals delivered by monocytes, but also generated 'off' signals to T cells. Mitogenic stimulation of T cells induces the synthesis of lymphokines such as IL-2, IFN-γ and the expression of the light chain (p55, CD25) of the IL-2R. The binding of IL-2 to its receptor induces signals which permit progression of T cells to S phase with subsequent proliferation. Our results indicate that the synthesis of IL-2 was not affected by ATG because (1) addition of recombinant IL-2 was unable to bypass the inhibition, and (2) increased concentrations of IL-2 were measured in culture supernatants. Conversely IFN-γ secretion as well IL-2Rα membrane expression were markedly reduced in the presence of ATG. Studies on mRNA expression revealed enhanced levels of IFN-γ and IL-2Rα chain mRNA whereas IL-2 mRNA level was not changed. The absence of intracellular accumulation of CD25 suggested that ATG blocked mRNA translation rather than the export of the protein from cytoplasm

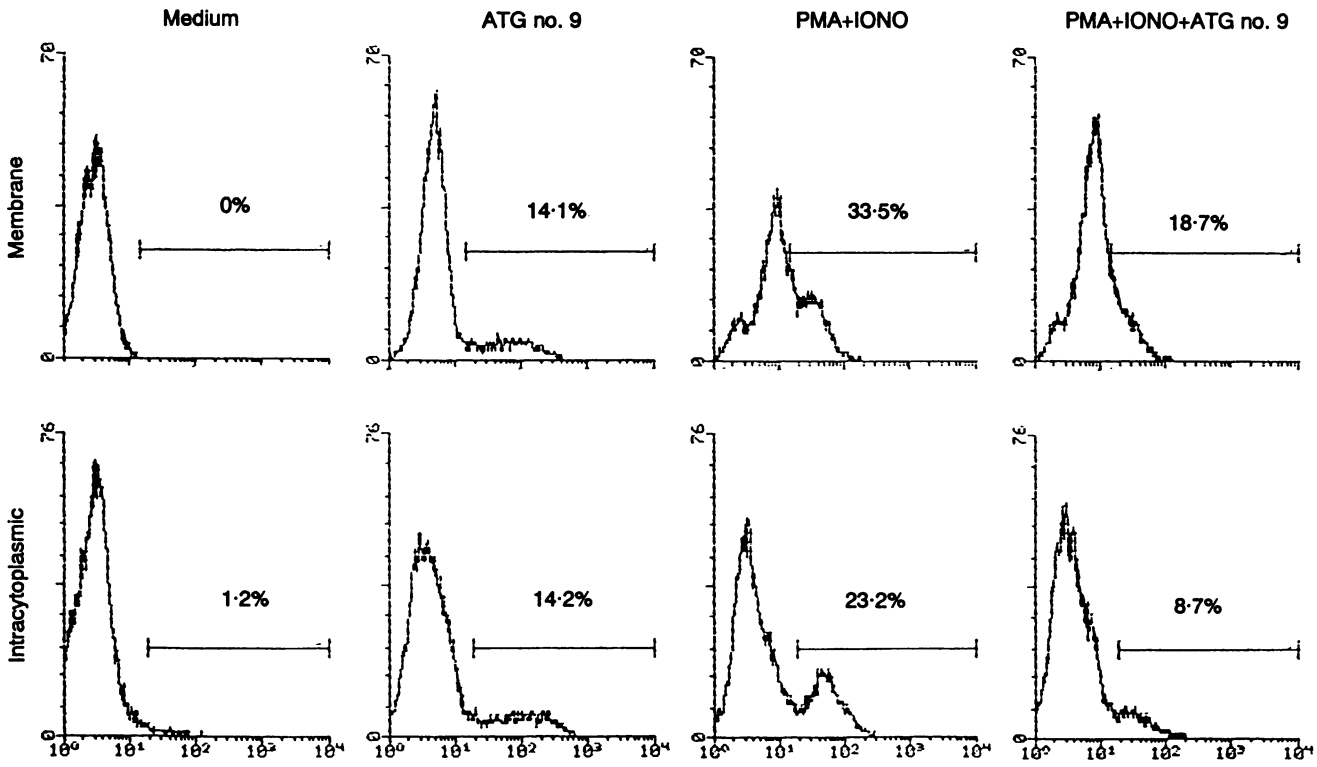

Figure 3. Comparative immunofluorescence analysis of membrane and intracytoplasmic CD25 expression on PMA plus ionomycin-activated lymphocytes in presence or absence of ATG. Cells were cultured with medium, ATG no. 9 (1·25 mg/ml), PMA (50 ng/ml) plus ionomycin (1 μg/ml) or PMA plus ionomycin and ATG no. 9. At the end of a 48-hr culture period cells were stained with FITC-anti-IL-2R mAb as described in Materials and Methods.

to membrane. We therefore concluded that inhibition of T-cell proliferation by ATG is primarily due to a post-transcriptional blockade of CD25 (IL-2Rα) expression.

As reported by different authors, IL-2R expression seems to be related to the activation of PKC. Stimulation of PKC by phorbol esters in the absence of other signals was reported to be sufficient to stimulate IL-2R expression on human resting peripheral blood lymphocytes.[17,18] This was confirmed by the use of H7, an inhibitor of protein kinase C, which blocked IL-2R expression (at a post-transcriptional level) without affecting IL-2 production in primary murine T-cell proliferation.[19] On the other hand, Mills and co-workers demonstrated that increase of cytosolic-free calcium concentration was required for synthesis of IL-2 but not for IL-2Rα expression.[20] In keeping with these reports our results suggest that ATG block T-cell proliferation by interfering with a PKC-induced signal, but not with a signal related to CA^{2+} mobilization. This hypothesis was further reinforced by evidence that ATG induced an increase of intracellular-free CA^{2+} in various cell lines (N. Bonnefoy-Berard, B. Verrier, C. Vincent and J. P. Revillard, manuscript in preparation.).

A large number of mAb directed against functional lymphocyte membrane molecules were reported to block T-cell proliferation. However to our knowledge only two mAb directed against CD4 molecule[21,22] and one against HLA class I antigen[23] have been shown to inhibit T-cell proliferation by preventing CD25 expression without impairment of IL-2 secretion. Moreover there are no available data with regard to which mechanism (transcriptional or post-transcriptional) could account for inhibition of CD25 expression by those mAb. We have previously demonstrated that ATG contain several antibodies specific for various functional molecules of leucocytes.[9] Among these we detected antibodies directed against CD4 and β_2-microglobulin (β_2m) molecules. However neither CD4 (BL4) nor anti-β_2m (BE104) antibodies added alone at saturating concentration (10μg/ml) did block CD25 surface expression or impaired IL-2 secretion (data not shown).

The cyclic peptide CsA and the macrolide lactone FK506, bind to the T-cell cytoplasmic receptors immunophilins, and the drug–immunophilin complexes bind to and inhibit the activity of the phosphatase calcineurin[24,25] and inhibit the translocation of the pre-existing component of NFAT-1 from cytoplasm to the nucleus.[25,26] Other regulating sequences in 5′ of the IL-2 gene may also be affected by CsA and FK506, as for instance AP-3, Oct-1 and to a lesser extent kB.[27] These immunosuppressive compounds block IL-2, IL-3, IL-4, IFN-γ, tumour necrosis factor-α (TNF-α) and granulocyte–macrophage colony-stimulating factor (GM-CSF) synthesis at a transcriptional level, but do not affect IL-6, IL-8, TNF-β, tumour growth factor-β (TNF-β) or IL-2Rα mRNA expression.[28,29] Furthermore FK506 and CsA do not affect the increased expression of the integrins (LFA-1, VLA-4, VLA-5 and VLA-6), which depends on the transport of preformed molecules but not on *de novo* synthesis.[30] In contrast, RPM does not inhibit IL-2 synthesis and CD25 (IL-2Rα) expression, but appears to block the intracellular signal(s) generated by the binding of IL-2 to its receptor which allow(s) the progression into the S/G2 phase of the cell cycle. IL-4-driven T-cell proliferation is resistant to RPM.[7]

Thus the post-transcriptional inhibition of CD25 (IL-2Rα) by ATG and the dissociation between decreased IFN-γ and unimpaired IL-2 secretion appear to be distinct from the mechanisms by which other immunosuppressive agents can inhibit T-cell activation and proliferation. Since the ATG concentrations required to induce the blocking effects described in the present report are achieved in plasma during ATG treatment by infusions of 10–20 mg/kg body weight, it is suggested that ATG may synergize with other immunosuppressive agents in combined treatment protocols.

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