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Antigen presentation by astrocytes primes rat T lymphocytes for apoptotic cell death

A model for T-cell apoptosis *in vivo*

R. Gold,[1*] M. Schmied,[3,4*] U. Tontsch,[2] H. P. Hartung,[1] H. Wekerle,[2] K. V. Toyka[1] and H. Lassmann[3]

[1]*Department of Neurology, Clinical Research Unit for Multiple Sclerosis, Julius-Maximilians-Universität, Würzburg, the* [2]*Department of Neuroimmunology, Max Planck Institute for Psychiatry, Martinsried, Germany, the* [3]*Research Unit for Experimental Neuropathology, Austrian Academy of Sciences and the* [4]*Division of Neurorehabilitation, Department of Neurology, University of Vienna, Vienna, Austria*

**These authors contributed equally*

Correspondence: Dr Ralf Gold, Neurologische Universitätsklinik, Josef-Schneider-Straße 11, D-97080 Würzburg, Germany

Summary

In this study we have characterized apoptotic cell death of autoreactive T cells resulting from their interaction with astrocytes and the modulatory effect of steroid hormones. Time kinetics of T-cell activation by interferon (IFN)-γ-treated astrocytes from neonatal Lewis rats and by professional antigen presenting cells (APCs) from bulk suspensions of thymus or spleen were performed. [^{3}H]Thymidine incorporation of neuritogenic P_2- and encephalitogenic myelin basic protein (MBP)-specific T-cell lines declined after 48 h in culture with astrocytes. A similar suppressive effect was observed when T cells were cocultured with thymic APCs and astrocytes. This effect disappeared when astrocytes were separated by a transwell system. After 72 h of culture with astrocytes a mean of 17.5±12.4% T cells exhibited morphological signs of apoptosis. Apoptosis was identified by light microscopy, and confirmed by electron microscopy, by in situ *tailing reaction and by agarose gel electrophoresis. Glucocorticosteroids and oestrogen specifically enhanced T-cell apoptosis within 8 h (69.8±23.1% and 69±17.1%, respectively) when added after 72 h to the astrocyte system, but not at earlier time points of T-cell activation or when thymic APCs were used. Glucocorticoid-mediated T-cell apoptosis was inhibited by the steroid-receptor antagonist RU 486. Pregnenolone, lipocortin-1, indomethacin and transforming growth factor-β did not induce apoptosis in this system. The steroid effect was not associated with CD28, IL-2 receptor, or transferrin-receptor expression, which were equally upregulated on T cells activated by astrocytes or thymic APC as shown by fluorescence activated cell sorting (FACS) analysis. We conclude that astrocytes as CNS-specific APC may render T cells susceptible for induction of apoptotic cell death. Some steroid hormones can markedly enhance this process* in vitro *and may augment an additional effect of a systemic corticosteroid response* in vivo *during recovery from autoimmune encephalomyelitis.*

Keywords: apoptosis; encephalomyelitis; glucocorticoids; multiple sclerosis

Abbreviations: APC = antigen presenting cell; EAE = experimental autoimmune encephalomyelitis; FACS = fluorescence activated cell sorting; IFN = interferon; MBP = myelin basic protein

Introduction

Apoptotic cell death of T lymphocytes is now being considered a major mechanism of clearance of inflammatory lesions in experimental autoimmune encephalomyelitis (EAE) (Pender *et al.*, 1991; Schmied *et al.*, 1993). The cellular or humoral signals mediating T-cell apoptosis in the CNS have not yet been identified. There is evidence that apoptosis does not represent a general phenomenon of immune regulation, but appears to be rather organ-specific. In EAE, apoptosis of T cells is mainly seen at the site of CNS inflammation, but not at the dermal sensitization site and only rarely in lymph nodes or spleen (Schmied *et al.*, 1993). Findings in other autoimmune diseases also favour a role of tissue-specific

factors: in experimental allergic neuritis, a T-cell mediated autoimmune disease of the PNS, T-cell apoptosis was found at a much lower rate than in EAE (Zettl *et al.*, 1994). Moreover, apoptosis of T cells in bioptical material from inflamed human muscle or skin is a rare event (R. Gold, M. Schmied and H. Lassmann; unpublished observations).

It is conceivable that adjacent cells such as astrocytes and microglial cells directly or through soluble factors render T cells susceptible for apoptosis or that systemic humoral factors known to have an immunoregulatory potential are instrumental in triggering apoptosis of T lymphocytes. In this study we investigated two pro-apoptotic mechanisms. Astrocytes are partially competent APCs, that are not able to trigger the complete T-cell activation programme (Matsumoto *et al.*, 1993; Weber *et al.*, 1994). We studied antigen-driven effects of astrocytes on T cells. Secondly, we evaluated the role of steroid hormones which can be produced locally (Hu *et al.*, 1987), or released systemically during the course of EAE (MacPhee *et al.*, 1989; Mason, 1991), and of other immunosuppressive factors in modulating T-cell apoptosis.

Material and methods

Cells and culture

All cell culture media and supplements were obtained from GIBCO (Eggenstein, Germany).

Cycloheximide, $ZnSO_4$, indomethacin, estradiol, dexamethasone, pregnenolone and hydrocortisone were supplied by Sigma (Deisenhofen, Germany). Sterile stock solutions of the steroid hormones were prepared in alcohol, filtrated and kept at –20°C until use. N^G-monomethyl L-arginine was obtained from Calbiochem (Frankfurt, Germany). Natural bovine lipocortin-1 and RU 486 were kind gifts of Dr R. Blake Pepinsky and of Dr S. Schwarz. Lyophilized recombinant rat IFN-γ (Holland Biotechnology; Leiden, The Netherlands) and human transforming growth factor-β (Genzyme, Cambridge, Mass., USA) were reconstituted and stored at –80°C until use.

Myelin basic protein- and P_2-specific CD4 positive rat T-cell lines were established as described previously (Gehrmann *et al.*, 1992; Jung *et al.*, 1992). Briefly, female Lewis rats (Zentralinstitut für Versuchstierzucht, Hannover, Germany) were immunized in the hind footpad with 100 μg MBP or 200 μg P_2 protein emulsified in complete Freund's adjuvant (DIFCO, Detroit, Mich., USA). All immunizations were performed according to Bavarian state regulations. Ten days later draining lymph nodes were removed and a single-cell suspension was prepared. Lymph node cells were then cultured at a density of 8×10^6 cells/ml in 60-mm plastic dishes (Nunc, Wiesbaden, Germany) in the presence of 20 μg ml^{-1} P_2 or 10 μg ml^{-1} MBP in RPMI-1640 supplemented with 1% normal rat serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM glutamine. Antigen-specific T cells were selected by repeated cycles of propagation in medium enriched with 7.5% supernatant of concanavalin A-treated mouse spleen cells and 10% horse serum instead of rat serum, followed by antigen-specific restimulation using irradiated (4000 rad) syngeneic thymus cells. For all experiments T-cell lines were taken from the same restimulation cycle.

Astrocytes were isolated from brain of newborn Lewis rats as described previously (Tontsch and Bauer, 1991). They were cultured in 75-cm^2 plastic flasks (Nunc) in basal medium Eagle's supplemented with 10% foetal calf serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Purity of the cells, which were used for the experiments between 4 and 6 weeks *in vitro* to ensure homogenous I-A expression, was confirmed by FACS analysis using antibodies against glial fibrillary acidic protein (Dako, Hamburg, Germany) and OX-42 (Dianova, Hamburg, Germany) recognizing a microglial surface antigen. For glial fibrillary acidic protein analysis astrocytes were fixed and permeabilized in 4% paraformaldehyde and 0.025% Nonidet-P40 as described previously (Gold *et al.*, 1994). With both of these antibodies, cultures were found to be of >98% purity (not shown).

Surface expression of I-A molecules was monitored by FACS analysis using the mouse monoclonal antibody OX-6 (specific for non-polymorphic rat I-A molecule) purified from the supernatant of hybridoma cells (ATCC, Rockville, Md, USA).

Fibroblast cultures were established from rats skin after preincubation with collagenase. Purity of the cultures was monitored by analysing expression of the Thy 1.1 antigen using the monoclonal antibody OX-7 (Camon, Wiesbaden, Germany).

T-cell activation and proliferation studies

For FACS analysis of surface expression of IL-2 receptor, transferrin receptor and CD28 T cells were separated from stimulating astrocytes or from thymic APCs by gradient centrifugation. They were then incubated with the respective monoclonal antibodies OX-39 and OX-26 (both from Dianova), or clone JJ319 [kind gift of Professor T. Hünig, Würzburg (Tacke *et al.* 1995)] and processed as described by Jung *et al.*, (1992).

For flow cytometric evaluation of DNA content the propidium iodide method was used. Samples were treated as described by Tabi *et al.* (1994).

Resting P_2- or MBP-specific T cells were added at 4×10^4 per well together with 10^6 irradiated thymic or 3×10^5 splenic APCs, 10^4 IFN-γ pretreated and irradiated astrocytes, or both types of APCs, and antigen in 100 μl medium in 96-well flat-bottom microtitre plates (Nunc). The optimal number of astrocytes for T-cell activation studies was titrated beforehand. Triplicate cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cultures were pulsed with 0.2 μCi [^{3}H]thymidine per well (Amersham-Buchler, Braunschweig, Germany) for 16 h and harvested at the indicated time points. The cells were collected on glass fibre filter paper by a Betaplate 96-well harvester (Pharmacia-

LKB, Turku, Finland). The radioactivity associated with the dried filter was quantified with a 96-well Betaplate liquid scintillation counter (Pharmacia-LKB).

For trans-well studies, astrocytes were seeded in 24-well plastic dishes (Nunc®). T-cell activation by thymic APCs was performed in Falcon tissue culture inserts with a permeable membrane of 0.45 μm pore size (Becton-Dickinson, Heidelberg, Germany) inserted into the same well. On day 2, T cells and APCs were carefully removed from each insert, split up and distributed into three wells of a 96-well flat bottomed dish (Nunc)

Quantification of apoptosis

P_2 or MBP-specific T cells (5×10^5) were activated by APCs (astrocytes, thymocytes or spleen cells) that were seeded on glass coverslips inserted into a 35-mm plastic dish (Nunc). Cocultures were fixed by carefully removing the culture medium, air-drying and subsequent treatment with Carnoy's fixative for 5 min at room temperature. Coverslips were dried and stored at room temperature. For morphological assessment of apoptosis (Schmied *et al.*, 1993) one half of the coverslip was stained with Giemsa's reagent, and 1000 T cells per sample were analysed.

For enzymatic labeling with *in situ* nick translation or tailing, the remaining half of the coverslip was rinsed twice with a 0.05 M TRIS buffer at pH 7.2. It was then incubated for 1 h with 50 μl of a reaction mixture containing 1 μl digoxigenin-labelled nucleotides (Dig DNA labelling mixture; Boehringer, Mannheim, Germany) and 5 U DNA polymerase I (New England Biolabs, Boston, Mass., USA) or 12 U terminal deoxynucleotidyl transferase (Promega, Heidelberg, Germany) in the appropriate buffer as described before (Gold *et al.*, 1994). The reaction was stopped by adding 0.5 M EDTA. Sections were then treated with an alkaline phosphatase-labelled anti-digoxigenin antibody (Boehringer) at a dilution of 1:600 for 1 h. Colour reaction was visualized by alkaline phosphatase cytochemistry using NBT/BCIP (Boehringer) as a chromogen. Cultures were rinsed and embedded in oil or Aquatex (Merck, Darmstadt, Germany).

Other cultures were fixed with 3% buffered glutaraldehyde, osmicated and embedded in plastic according to routine procedures. Thin sections were stained with uranyl acetate and lead citrate and analysed on a Jeol 100S electron microscope.

DNA gel electrophoresis

For DNA isolation, T lymphocytes were removed from cocultures by careful rinsing in order not to detach astrocytes, centrifuged at 150 *g* and then processed essentially as described by Miller *et al.* (1988). Before DNA precipitation, the aequous phase was diluted to give a final NaCl concentration of 0.3 M. Two and a half volumes of ethanol were added and incubated on ice for 1 h to allow precipitation

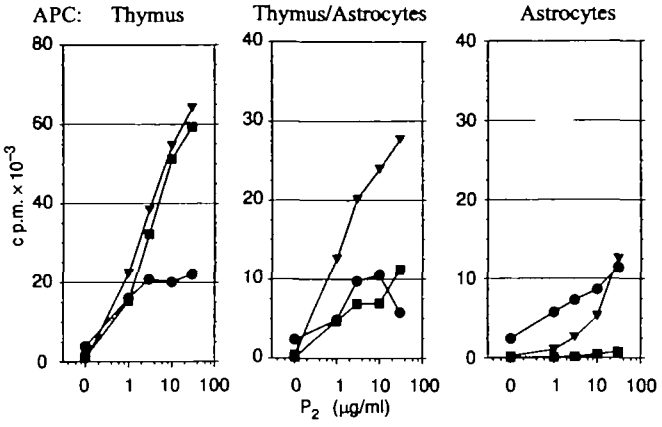

Fig. 1 Time kinetics of T-cell proliferation with different types of APCs: irradiated thymocytes, IFN-γ pretreated astrocytes and thymocytes in the presence of unstimulated astrocytes. Cultures were set up in parallel, pulsed with [^{3}H]thymidine for 16 h and harvested at the different time points after activation indicated by closed circles (day 1), closed triangles (day 2) and closed squares (day 3). The *x*-axis shows titration of the antigen P_2. The *y*-axis denotes mean counts per minute of triplicate cultures. Note the different scale of the *y*-axis for thymus APCs.

of smaller fragments. DNA was recovered after high speed centrifugation, washed in 70% ethanol and dissolved in TE buffer (Sambrook *et al.*, 1989). Concentration of DNA was determined spectrophotometrically. Three micrograms of DNA per lane and 1 μg of a molecular weight standard (λDNA–*Hin*dIII digest; Pharmacia, Freiburg, Germany) were loaded into slots. Electrophoresis was carried out in a stepwise (0.7%/1.2%) agarose gradient gel at 100 V for 90 min. DNA was visualized with ethidium bromide.

Statistical evaluation

Statistical analysis was performed using Student's *t* test and ANOVA test as provided by the SPSS 4.0 computer program (SPSS Inc., Chicago, Ill., USA). $P < 0.05$ and $P < 0.01$ were considered statistically significant.

Results

Different pattern of T-cell activation by astrocytes and thymic APCs: inhibitory effect of astrocytes

Astrocytes activated P_2-specific T cells (Fig. 1) in a dose-dependent manner in the presence of antigen, but were clearly less efficient than thymic APCs. With professional thymic APC a strong proliferative response was seen on day 2 after stimulation had started, and remained elevated therafter (Fig. 1), whereas T-cell activation by astrocytes almost completely ceased on day 3. The presence of either untreated (Fig. 1) or IFN-γ pretreated astrocytes also inhibited T-cell activation. Inhibition of nitric oxide synthase by N^G-monomethyl L-arginine did not restore the ability to induce T-cell proliferation (data not shown). Similar results were

Table 1 *T-cell apoptosis after antigen presentation by thymus cells or astrocytes*

	Medium alone		Hydrocortisone (t = 0 h)		Hydrocortisone (t = 72 h)	
Time of analysis (hours)	48	72	48	72	72	72 + 8
Thymus APC	3.2	2.8	6.5	4.5	0	5
Astrocyte	1.3	6.5	3.5	8	30	99

Apoptosis of autoreactive T cells after antigen-specific activation by different types of APC. Results are given as mean %±SD at each time point or treatment. t = 0 indicates that steroids were added at the beginning of the experiment, while t = 72 h indicates addition after 72 h. Apoptosis was evaluated after 48, 72 or 80 h. Controls for cultures in the right column (hydrocortisone t = 72 h) were analysed at the same time when steroids were added to assess basal values of apoptosis in untreated cells.

obtained with MBP-specific T-cell lines (data not shown). To further delineate whether the effect was dependent on a direct cellular contact or was humorally mediated, we conducted experiments in which astrocytes were separated from T cells and thymic APCs by a permeable membrane of a trans-well system. On day 2, T cells were transfered to microtitre plates, labelled, and harvested 1 day later. No inhibition was seen with astrocytes separated by a semi-permeable membrane, indicating that the effect is probably not mediated by soluble factors (data not shown).

To show that the effect is astrocyte specific we investigated the interaction of rat fibroblasts with antigen presentation by thymic APCs. Both untreated and IFN-γ pretreated fibroblasts had a strong inhibitory effect on T-cell activation, but this completely disappeared after addition of N^G-monomethyl L-arginine, indicating that it was mediated by nitric oxide (not shown).

T-cell apoptosis with different types of APCs and its modulation by hydrocortisone

The inhibition of T-cell proliferation may indicate T-cell anergy or death after stimulation by astrocytes. This was further investigated by comparing nuclear morphology of T cells stimulated with thymus APC and IFN-γ-pretreated astrocytes. Minor differences in T-cell apoptosis were found 72 h after activation, when most of the T cells were still viable (Table 1, left column; Fig. 2A). After stimulation with astrocytes only a small increase of T-cell apoptosis was noted resulting in a mean of 17.5%±12.4 T cells displaying morphological signs of apoptosis. We then tried to modulate T-cell apoptosis by adding hydrocortisone at a final concentration of 5×10^{-5} M, which was chosen on the basis of its suppressive effect on T-cell activation (data not shown) and *in vitro* studies by Cohen *et al.* (1970). This treatment was very effective in augmenting apoptosis when added 72 h after T-cell stimulation but not at earlier time points (Table 1, middle and right columns; Fig. 2B and D). Only T cells stimulated by astrocytes but not by thymic APCs were susceptible to steroid-induced apoptosis. A series of experiments in which hydrocortisone was added 72 h after T-cell activation by astrocytes is summarized in Fig. 3. After 3 h in culture with steroids, a significant increase of T-cell apoptosis was observed, which was further augmented after 8 h. This effect was not due to the alcohol that served as a solvent for hydrocortisone and did not increase T-cell apoptosis when added in equal amounts.

To show that the effect is steroid-specific, we studied hydrocortisone-mediated apoptosis in the presence of the steroid-receptor antagonist RU 486. In the presence of RU 486, there was a marked protection of T cells, only some of which underwent apoptosis (Fig. 2C). A series of inhibition experiments is summarized in Fig. 4. RU 486 alone had no effect on viability of T cells in control cultures when added at a final concentration of 10^{-5} or 10^{-7} M.

Apoptotic cell death was defined by morphological criteria (Wyllie *et al.*, 1980), i.e. cellular shrinkage with nuclear chromatin condensation and eventual collapse into patches along the nuclear membrane (Fig. 2E). DNA fragmentation could be demonstrated at the single cell level by using the method of *in situ* tailing (Fig. 2A and B). In these cultures there was a good correlation between morphological evaluation of apoptosis and assessment by *in situ* tailing. Nuclei of astrocytes were not labelled, nor did they display morphological signs of apoptosis. Finally, oligonucleosomal DNA fragmentation was proven by gel electrophoresis of DNA extracted from lymphocytes (Fig. 5). Steroid-induced apoptosis did not require protein synthesis, since addition of cycloheximide had no effect as shown by gel electrophoresis (Fig. 5) and in morphological studies with a mean of 48.1±24.9% in the presence of cycloheximide versus 54.3±25.7% in hydrocortisone-treated cultures. T-cell apoptosis could be inhibited by adding $ZnSO_4$ (61.8±27% versus 12±1.4% in the presence of $ZnSO_4$) indicating that the action of an endonuclease was required for DNA fragmentation.

Modulation of T-cell apoptosis by other steroid hormones and immunomodulatory compounds

Dexamethasone was as effective as hydrocortisone in inducing T-cell apoptosis when added at late time points

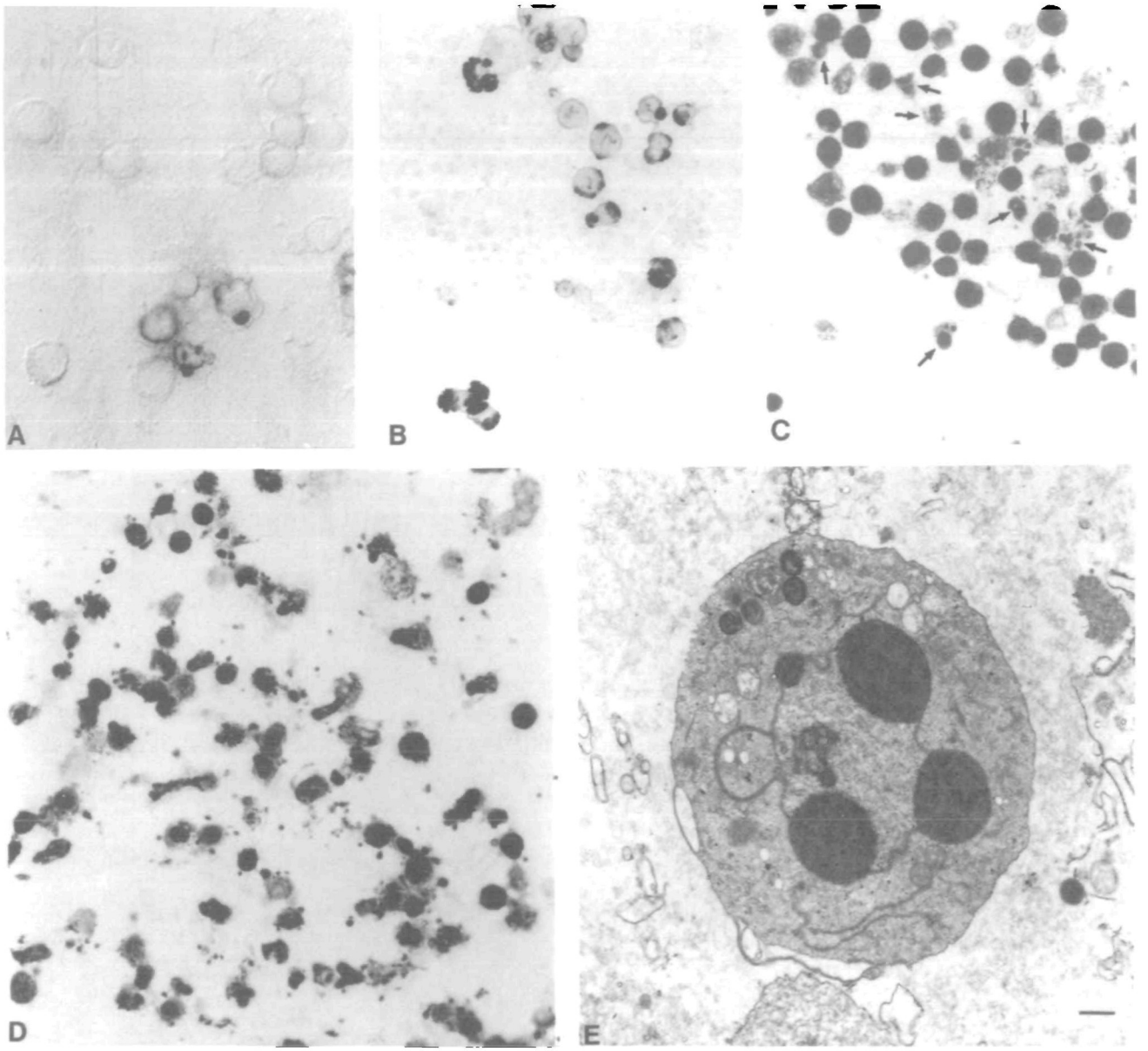

Fig. 2 Morphological evaluation of apoptotic T cells in different cultures. (**A**) The *in situ* tailing reaction was applied to a coverslip with T cells 72 h after activation by astrocytes. Only a few T cells underwent apoptosis, resulting in a positive signal within apoptotic bodies. The remaining intact T cells are not labelled. (**B**) After exposure to hydrocortisone (5×10^{-5} M) a significant increase of labelled apoptotic T cells could be detected by the *in situ* tailing reaction. (**C**) In the presence of RU 486 (10^{-5} M), a marked inhibition of hydrocortisone-mediated apoptosis was observed. Only few T cells display morphological signs of apoptosis and are marked by arrows (Giemsa stain). (**D**) The maximal induction of T-cell apoptosis was seen when cultures were fixed 8 h after addition of hydrocortisone, 72 h after T-cell activation by astrocytes (Giemsa stain). Magnification in **A–D**, ×350. (**E**) An electron micrograph of a cultured apoptotic T cell showing characteristical condensation of the nuclear chromatin and cellular shrinkage. The bar = 1 μm.

of activation (64±7% versus 5.5±2.1% in control cultures). A much lower concentration of dexamethasone was required, since this steroid has a higher binding affinity to the steroid receptor and does not bind to the cortisol-binding protein. Furthermore, oestrogen was equally powerful in inducing T-cell apoptosis when added at a concentration of 5×10^{-5} M (69±17.1% versus 26.2±9.6% apoptosis in control cultures at 72 h). Similar to hydrocortisone the effect of oestrogen could be inhibited by adding $ZnSO_4$. Pregnenolone, a key hormone for steroid biosysthesis and one of the brain derived steroids had no clear effect. Because of their powerful immunomodulatory action other substances, e.g. lipocortin-1 (3 μg ml^{-1}), transforming growth factor-β (10 ng ml^{-1}) and indomethacin (2 μg ml^{-1}), were tested. All of these failed to augment T-cell apoptosis.

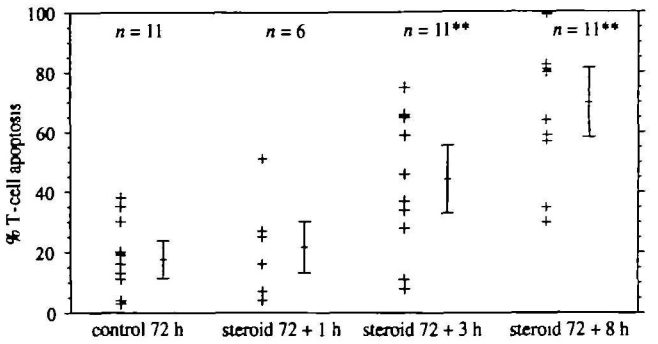

Fig. 3 Modulation of T-cell apoptosis by adding hydrocortisone (5×10^{-5} M) 72 h after activation of P_2-specific T-cell line by astrocytes as APCs. Controls did not receive hydrocortisone. *n* indicates the number of experiments. Results were obtained by evaluating nuclear morphology of Giemsa-stained T cells. For each experiment 1000 T cells were analysed. Vertical bars represent mean±SD. $^{**}P < 0.01$ compared with controls.

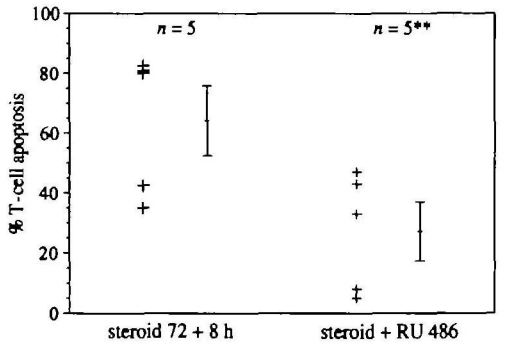

Fig. 4 RU 486 inhibits hydrocortisone-mediated T-cell apoptosis. Seventy-two hours after activation of P_2-specific T-cell line by astrocytes, APC cultures were treated with hydrocortisone (5×10^{-5} M) alone or together with RU 486 (10^{-5} M). Eight hours later cultures were fixed. *n* indicates the number of experiments. Results were obtained by evaluating nuclear morphology of Giemsa-stained T cells. For each experiment 1000 T cells were analysed. Vertical bars represent mean ±SD. $^{**}P < 0.01$ compared with controls.

Patterns of T-cell activation by astrocytes and thymic APCs

Next we examined whether the effects on apoptosis are linked to incomplete T-cell activation. Therefore we studied, by FACS analysis, activation marker molecules on T cells. There was no significant difference for up-regulation of IL-2 receptor (Fig. 6), transferrin receptor and CD28 (not shown) on T cells activated with thymus or astrocyte APCs.

We used the propidium iodide method to investigate the respective stage of the cell cycle in which T cells were left following stimulation by thymic APC or astrocytes. There was no difference detectable with regard to distribution in the cell cycle.

Discussion

Antigen presentation by astrocytes had two effects on T-cell activation: (i) there was incomplete T-cell activation after

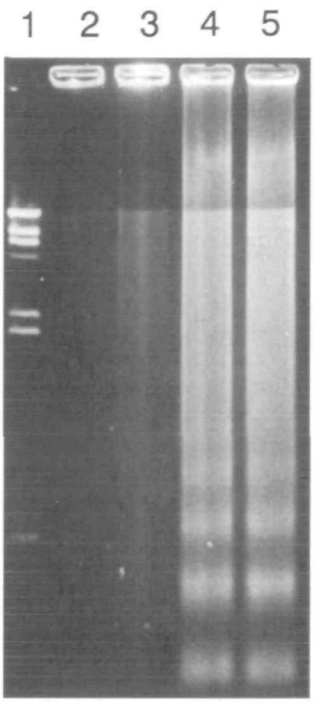

Fig. 5 DNA extracted from IL-2 treated lymphocytes (lane 2), T cells 72 h after astrocyte activation (lane 3), after addition of hydrocortisone for 8 h (lane 4) and hydrocortisone together with cycloheximide (lane 5) was electrophoretically separated in a stepwise agarose gel. Lane 1 shows the molecular weight marker λDNA–*Hin*dIII digest.

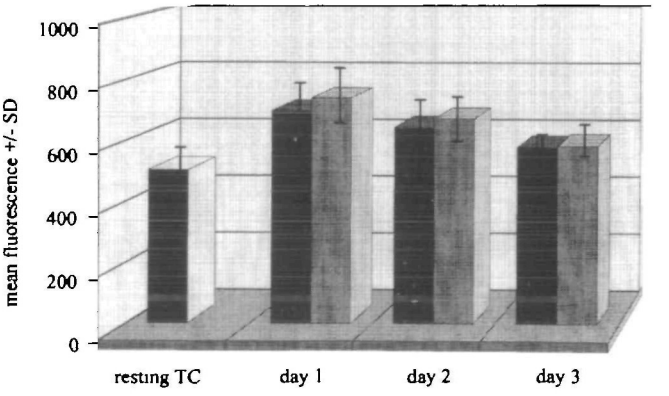

Fig. 6 Time kinetics of up-regulation of surface IL-2 receptor expression on T cells after stimulation by astrocytes or thymic APCs. At each time point T cells were separated from astrocytes or thymic APCs by gradient centrifugation. Values represent the mean fluorescence±SD from two experiments where 5000 events were analysed per sample. Dark shaded bars = astrocyte APCs; light shaded bars = thymus APCs.

day 2 as reflected by low thymidine incorporation in spite of elevated IL-2 receptor, transferrin receptor and CD28 expression; (ii) it increased susceptibility of T cells to undergo apoptotic cell death when steroid hormones were added to the culture.

The first effect, which was exerted by astrocytes even in the presence of thymus APCs, was dependent on a direct cellular contact and did not depend on prior stimulation of

astrocytes by IFN-γ. It disappeared when astrocytes were separated by a permeable membrane of trans-well inserts. Only rare exceptions have been noted, where unstable humoral factors like nitric oxide were quickly inactivated or scavenged (Blough and Zafiriou, 1985; Drapier *et al.*, 1991; Ignarro *et al.*, 1993), giving rise to false negative results in trans-well studies. However, this is unlikely in our system as addition of inhibitors for nitric oxide production did not augment T-cell proliferation. Similar suppressive effects of astrocytes on T-cell proliferation have been reported by Matsumoto *et al.* (1993) and by Weber *et al.* (1994), who showed that human astrocytes are only partially competent APCs, and by Meinl *et al.* (1994) who further characterized the immunomodulatory effects of human astrocytes. In these studies inhibition of prostaglandin synthesis by indomethacin had no effect on astrocyte-mediated immunosuppression (Matsumoto *et al.*, 1993) or caused a partial reversal of T-cell inhibition (Meinl *et al.*, 1994). Nor did neutralization of transforming growth factor-β 1 and 2 revert the inhibitory effect of rat or human astrocytes (Matsumoto *et al.*, 1993; Meinl *et al.*, 1994). In our system, these compounds did not modulate T-cell apoptosis. Fibroblasts also exerted a suppressive effect on T-cell activation. In contrast to astrocytes fibroblast-mediated inhibition disappeared after addition of N^G-monomethyl L-arginine, indicating the involvement of nitric oxide and rendering these results less significant than the non-nitric oxide-dependent inhibition by astrocytes.

Astrocytes but not thymic APCs primed T cells for steroid-mediated apoptosis. This has been demonstrated using a number of different approaches based on morphology, molecular labelling techniques and gel electrophoresis. This may, in part, explain the patterns of EAE *in vivo*, where T cells, activated outside the nervous system (Wekerle *et al.*, 1986; Ohmori *et al.*, 1992), may further interact with only partially competent local APCs in the brain, and finally are cleared from the CNS by apoptotic cell death (Pender *et al.*, 1991; Schmied *et al.*, 1993). Furthermore, raised systemic levels of corticosterone have been found during recovery from EAE (MacPhee *et al.*, 1989; Mason, 1991), where T-cell apoptosis reaches its maximum (Schmied *et al.*, 1993), and only low values of apoptosis are observed in adrenalectomized rats (Smith *et al.*, 1994). These two effects may synergize to cause apoptotic death of primed T cells in the CNS. Although most investigators agree that at least in rats, microglia are the cells that predominantly express MHC class II antigens *in vivo* (Hickey *et al.*, 1985; Matsumoto *et al.*, 1986; Vass *et al.*, 1986) some expression of I-A molecules on astrocytes has been observed in very severe inflammatory lesions (Hickey *et al.*, 1985) or after intrathecal injection of high doses of IFN-γ (Vass and Lassmann, 1990). Thus the latter studies suggest that MHC class II restricted antigen presentation by astrocytes may not only take place *in vitro* (Fontana *et al.*, 1984) but also *in vivo*.

In vitro studies have shown that susceptibility of mature T cells to steroid-induced apoptosis is restricted to distinct stages of the cell cycle, and T-cell activation by engagement of the T-cell receptor can abolish their susceptibility (Zacharchuk *et al.*, 1990; Tuosto *et al.*, 1994). This underscores our results that a distinct activational state of the T cell is required: T cells were only susceptible to steroid-induced apoptosis when hormones were added 72 h after activation, but not at earlier time points. Using the propidium iodide method, we detected no significant differences in cell cycle distribution of T cells after stimulation by astrocytes or thymic APC.

Another steroid hormone, oestrogen, also exhibited a marked effect on astrocyte-driven T-cell apoptosis. This is of particular interest with respect to its protective influence on EAE (Jansson *et al.*, 1994) and the protection from relapses of multiple sclerosis during pregnancy (Hutchinson, 1993). In contrast, pregnenolone was ineffective on T-cell apoptosis. This steroid has been identified in rat brain (Corpechot *et al.*, 1981) and can be synthesized and further converted in glial cells (Hu *et al.*, 1987; Kabbadj *et al.*, 1993). Neither did lipocortin-1, a mediator of glucocorticosteroid action (Flower and Rothwell, 1994) which is also synthesized in cultured astrocytes (Gebicke-Haerter *et al.*, 1991), increase T-cell apoptosis in spite of its immunosuppressive effect on T-cell proliferation (R. Gold, R. B. Pepinsky, H.-P. Hartung, unpublished results). Transforming growth factor-β, another potent immunosuppressive cytokine (Roberts and Sporn, 1990) which induces apoptosis of murine T-cell clones (Weller *et al.*, 1994), was also found to be devoid of any effect in our system.

At present the priming mechanism of astrocytes remains elusive. A suppressive effect of some astrocyte clones on T-cell proliferation has been described earlier (Wekerle *et al.*, 1987). We could exclude a contribution of surface activation markers, since they were equally upregulated after antigen presentation by astrocytes and thymic APC. Other candidates that could modulate T-cell apoptosis comprise the action of co-stimulatory cytokines like IL-1 (Snyder and Unanue, 1982; Bergsteinsdottir *et al.*, 1991) or IL-2 (Nieto and Lopez-Rivas, 1989), the lack of co-stimulatory molecules on non-professional APCs (Nickoloff and Turka, 1994), altered intracellular signalling (Ullman *et al.*, 1990) and expression of genes associated with T-cell apoptosis (Osborne and Schwartz, 1994). Furthermore, autocrine T-cell suicide mediated by up-regulation of *Fas* or its ligand on astrocyte-activated T cells must be considered (Dhein *et al.*, 1995).

The efficacy of glucocorticosteroid treatment in patients with immune-mediated disorders of the nervous system has been attributed to their pleiotropic anti-inflammatory actions [for reviews, *see* Goldstein *et al.* (1992) and Haynes (1992)]. Our data raise the possibility that infiltrating T cells within the nervous system are susceptible to corticosteroid action which, as a consequence, augments their elimination by apoptosis. This has been shown in experimental autoimmune neuritis (Zettl *et al.*, 1995) and may be a central mechanism of high-dose glucocorticosteroid therapy in multiple sclerosis (Beck *et al.*, 1993).

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