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Establishment and characterization of a permanent T-cell line producing an antigen non-specific suppressor factor

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Summary. A permanent cell line designated SL4c has been established from a primary culture of murine BALB/c spleen cells regularly stimulated with large doses of irradiated allogeneic cells plus exogenous interleukin-2 (IL-2). After 8 months of cultivation, the cells of the SL4c line proliferate spontaneously and do not respond with an increase in proliferation to alloantigenic stimulation. The cells have the Lyt 1.2^+, Lyt 2.2^-, $L3T4a^+$, Thy 1.2^+ phenotype and exert a strong suppressive effect upon stimulation with freshly explanted cells. The SL4c line produces a suppressor factor (SF4c), which inhibits the mitogen-induced proliferation of normal lymphoid cells but does not suppress the proliferation of fibroblasts and sarcoma cells. The suppression is antigen non-specific, is not limited by H-2 restriction nor by interspecies barrier, and is not due to cytotoxic effect. However, the suppression is only detectable if the SF4c is added to the stimulated cells during the early stages of mitogen-induced proliferation. A tentative characterization of the relative molecular weight (MW) of the suppressor molecule based upon fractionation of SF4c supernatant on a Sepharose 6B column shows that the inhibitory activity is confined to the high MW fractions (300,000–350,000). Translation material obtained from *Xenopus laevis* oocytes, which were injected with RNA preparations isolated from SL4c cells, also shows the suppressive effect.

Abbreviations: C, complement; Con A, concanavalin A; FCS, fetal calf serum; IL-2, interleukin-2; LPS, lipopolysaccharide; mAb, monoclonal antibodies; 2-ME, 2-mercaptoethanol.

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INTRODUCTION

Techniques used to establish permanent T-cell lines make it possible to recognize the existence of the cell populations that are present in relatively low proportions in the organism. *In vitro* cultures of such cells open the way to studying the mechanisms of their effect. Cell lines capable of either stimulating or inhibiting the immune reactions, both in the antigen-specific and antigen non-specific manner, have been described (Schreier *et al.*, 1980, Fresno *et al.*, 1981, Aune & Pierce, 1984). Specific antisera and monoclonal antibodies against differentiation antigens disclosed the remarkable phenotype variability of immunoregulatory T-cell populations and the complexity of suppressor circuits (Green, Flood & Gershon, 1983, Eardley *et al.*, 1978). Preparation of monoclonal regulatory factors in sufficient quantities provides useful material for biochemical analyses and for study

of the mechanisms of action (Tanguchi *et al.*, 1982, Taussig & Holliman, 1979).

Here, we describe the establishment of a T-cell line that carries the phenotype markers of helper/inducer lymphocytes and produces a factor capable of inhibiting the activation of lymphocytes. Suppression is antigen non-specific, is not H-2 restricted, and is mediated by a factor associated with the high MW fraction of the culture medium. The cell line described here may represent a new component of immunoregulatory circuits.

MATERIALS AND METHODS

Animals

Mice of inbred strains BALB/c, C57BL/10SnSc (B10) and A.SW, and Lewis strain rats were used when 2–4 months old. Mice were maintained at the breeding unit of the Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague. Rats were obtained from the Institute of Physiology of the Czechoslovak Academy of Sciences.

Monoclonal antibodies

Cytotoxic monoclonal antibodies (mAb) of specificities anti-Thy 1.2 (Lake *et al.*, 1979), anti-Lyt 1.2 (Mark *et al.*, 1982) and anti-Lyt 2.2 (Gottlieb *et al.*, 1980) in the form of ascites fluids were used at a dilution of 1:100. MAb GK1.5 (Dialynas *et al.*, 1983) recognizing the L3T4a determinant was used in the form of culture supernatants diluted 1:10.

Preparation of interleukin-2 (IL-2)

Rat spleen cells at a concentration of 8×10^6/ml RPMI-1640 medium supplemented with antibiotics (penicillin, streptomycin), 5×10^{-5} M 2-mercaptoethanol (2-ME) and 10% fetal calf serum (FGS; Gibco Europe, East Molesey, Surrey, U.K.) were incubated with 5 μg/ml of concanavalin A (Con A; Sigma, St Louis, MO). After 48 hr, the cells were centrifuged for 10 min at 1000 ***g***, and the supernatants were stored at $-20°$ until used.

Establishment of the SL4c cell line and cloning

Spleen and lymph node cells from BALB/c male mice (2×10^6 cells/ml) were incubated with irradiated B10 cells (5×10^6 cells/ml, radiation dose 20 Gy from a ^{60}Co source) in RPMI-1640 medium supplemented with antibiotics, 2-ME (5×10^{-5} M) and 10% FCS (Gibco). Cultures were set up in a total volume of 5 ml in upright tissue culture flasks (No. 3013F, Falcon). After 7 days of incubation at 37° in an atmosphere of 5% CO_2, the cells were resuspended, and approximately 3 ml of the cell suspension was replaced with fresh medium containing irradiated B10 cells (20×10^6 cells per flask) and IL-2 (final concentration 10%). Afterwards, 3 ml of the medium were replaced every 3–4 days with fresh medium containing 10% IL-2, and $15–20 \times 10^6$ irradiated B10 cells were added to the cultures at 7-day intervals.

After 5 months of cultivation, the line was cloned by single-cell manipulation. Individual cells were seeded in wells of a flat-bottomed 96-well microplate (No. 3072, Falcon) in a volume of 0·2 ml of culture medium containing 5% IL-2 and 2×10^5 irradiated B10 cells per well. Growing colonies were transferred to 24-well plates (No. 3047, Falcon) and subsequently to culture flasks.

The growth of SL4c cells was tested for the dependence on alloantigenic stimulation and addition of exogenous IL-2. The cells did not proliferate in cultures without exogenous IL-2, but they grew well in cultures not supplemented with irradiated allogeneic cells.

Cytotoxic assay

For phenotype testing, SL4c cells prelabelled with sodium ^{51}Cr-chromate (Radiochemical Centre, Amersham, Bucks, U.K.) were incubated with mAb and complement (C; rabbit serum preabsorbed with mouse tissues) for 45 min at 37°. After incubation, ^{51}Cr release was assessed in the supernatant and expressed as a percentage of total activity.

In order to test the possible cytotoxic effect of SL4c cells, ^{51}Cr-labelled target cells (spleen cells, Con A blasts or EL4 cells, 2×10^4 cells/well) and the tested SL4c cells at varying ratios were incubated in round-bottomed 96-well microtitre plates (Titertek, Flow Laboratories, Irvine, Ayrshire, U.K.). After an appropriate incubation period (2 or 4 hr), the cells were centrifuged and the percentage of the tracer released into supernatant was determined.

Separation of cell populations

B cells were prepared by injecting BALB/c mice intraperitoneally with 0·5 ml of anti-Thy 1.2 mAb. Spleen cell suspension was obtained 24 hr later and was incubated in a nylon-wool column according to the method of Julius, Simpson & Herzenberg (1973). Nylon-adherent cells were collected and treated *in vitro* with anti-Thy 1.2 mAb plus C as described above.

T cells were isolated by incubation of BALB/c spleen and lymph node cells in a nylon-wool column (Julius *et al.*, 1973). The suspension of non-adherent cells was incubated in petri-dishes (Falcon) coated with swine anti-mouse immunoglobulin according to Wysocki & Sato (1978). After 60 min, the dishes were gently washed and the non-adherent cells were collected.

Mitogen-induced cell proliferation

Either unseparated lymphoid cells, or isolated T- or B-cell populations, were suspended in culture medium and adjusted to a concentration of 1×10^6 cells/ml; aliquots of 0·2 ml/well were incubated in 96-well flat-bottomed culture plates (No. 3072, Falcon), and Con A (Sigma) or bacterial lipopolysaccharide (LPS) was added to make a final concentration of, respectively, 5 and 40 μg/ml. Cell proliferation was assessed by adding 1 μCi [^{3}H]thymidine (specific activity 22·8 Ci/mmole, Institute for Research, Production and Application of Radioisotopes, Prague) per well for the last 8 hr or 16 hr of a 72-hr incubation period.

Molecular weight determination

Eight-ml supernatant samples from SL4c cell cultures were fractionated on Sephacryl S-200 or Sepharose 6B (Pharmacia, Uppsala, Sweden) columns, and individual fractions were tested for their efficacy to inhibit Con A- or LPS-induced proliferation of BALB/c lymphoid cells.

Preparation of RNA and its translation in Xenopus laevis *oocytes*

Total RNA was extracted from SL4c cells by the guanidine thiocyanate method according to Chirgwin *et al.* (1979). An equal volume of RNA was injected into *X. laevis* oocytes as described by Gurdon *et al.* (1971). Ten oocytes were injected with RNA (0·03 μl of RNA/oocyte, concentration of RNA 1 mg/ml) and incubated in 5 μl of sterile Barth's medium at 20°. Oocytes injected with water were incubated in parallel as a control. After 48 hr, the oocyte medium was removed, centrifuged and tested for suppressive activity.

RESULTS

Suppressive effects of SL4c cells and SF4c supernatant

Cells of the SL4c line were added to the cultures of BALB/c lymphoid cells stimulated with Con A, and

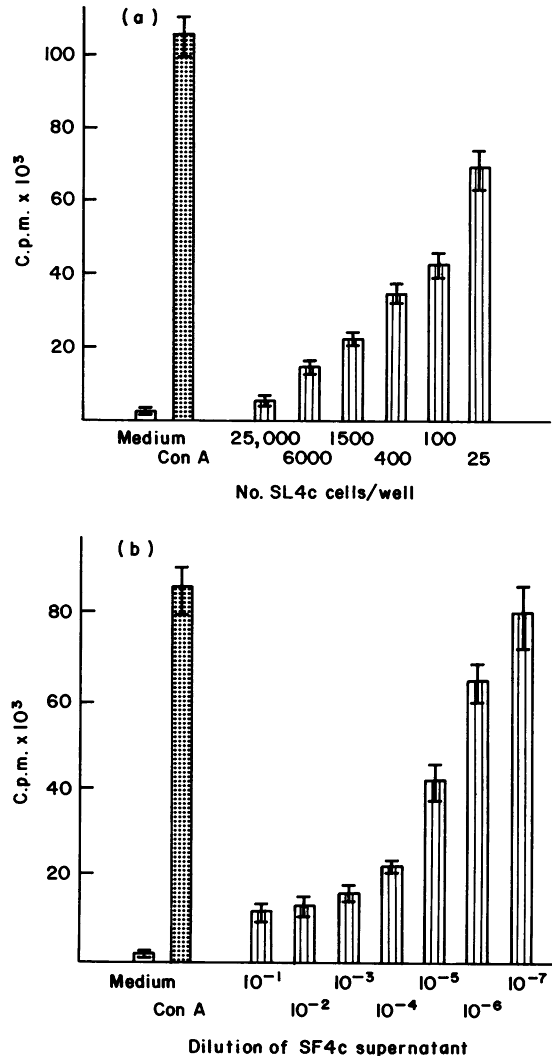

Figure 1. Suppressive effect of (a) SL4c cell line and (b) SF4c supernatant. SL4c cells in gradually decreasing numbers or SF4c supernatants at varying dilution ratios were added to 3×10^5 BALB/c spleen cells/well stimulated with Con A, and cell proliferation was assesed after 72 hr of incubation.

proliferation was assessed after 72 hr of incubation. The results in Fig. 1a show that SL4c cells strongly inhibited cell proliferation. Suppression was significant, even at concentration lower than 25 SL4c cells/well.

A suppressive effect similar to that obtained with SL4c cells was also demonstrated with supernatants from 3-day cultures of SL4c cells (initial concentration 1×10^5 cells/ml). In this case, cell proliferation was

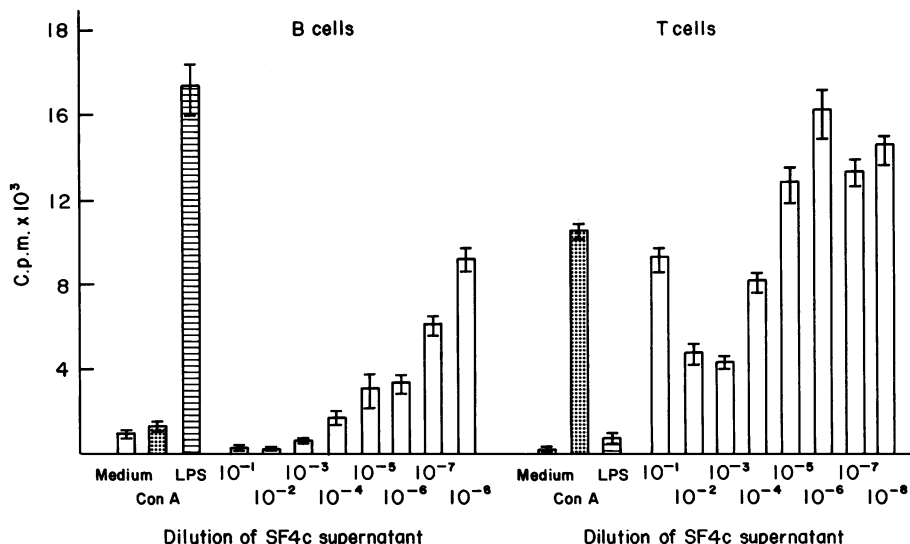

Figure 2. Suppression of B- and T-cell proliferation by SF4c supernatant. Purified B and T cells from BALB/c mice were stimulated with LPS or Con A in the presence of SF4c supernatant, and cell proliferation was assessed after 72 hr.

suppressed, even beyond the final dilution of $1:10^6$ (Fig. 1b).

Suppression of proliferation of T- and B-cell populations

Lymphoid cells from BALB/c mice were separated into T- and B-cell populations. The LPS-induced proliferation of B cells was completely inhibited by SF4c supernatants, and the suppressive effect was significant, even at a dilution of $1:10^8$ in this particular experiment (Fig. 2). The suppressive titres of SF4c on LPS-induced proliferation of B cells were usually $1:10^6$–$1:10^{10}$. On the other hand, proliferation of the Con A-stimulated population enriched for T cells was

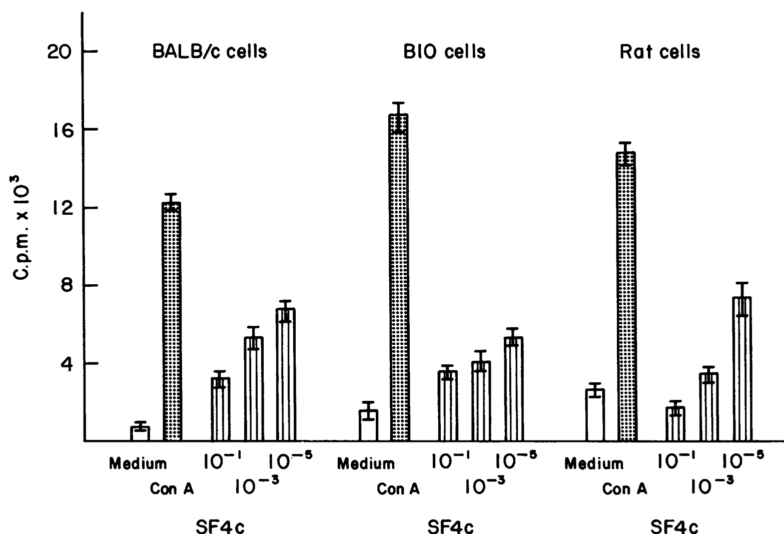

Figure 3. Suppressive effect of SF4c supernatant is not restricted by H-2 or interspecies barrier. Spleen/lymph node cells from BALB/c or B10 mice, or from Lewis rats, were stimulated with Con A in the presence of SF4c supernatant. Cell proliferation was assessed by adding [^{3}H]thymidine for the last 8 hr of the incubation period.

only partly inhibited (Fig. 2), and the effect was detectable at a much lower titre than that effective in the case of B lymphocytes. The suppressive effect of SF4c and partially purified T cells varied according to the effectiveness of T-cell purification and was very low, if any, with highly purified T cells. In some cases, there was a prozone effect (i.e. the suppression was more profound with more diluted than undiluted supernatant).

Effect of SF4c shows neither H-2 nor interspecies restriction

The suppressor factor released into supernatants from cultures of SL4c cells, which is a cell line of BALB/c (H-2^d) origin, inhibited proliferation not only of syngeneic BALB/c cells, but also allogeneic cells of B10 origin (H-2^b), and even proliferation of rat lymphoid cells (Fig. 3).

SF4c supernatant does not inhibit proliferation of non-lymphoid cells

Supernatants from SL4c cell cultures which strongly inhibited proliferation of mouse and rat lymphoid cells did not suppress proliferation of methylcholanthrene-induced murine sarcoma cells (MCll cells); neither did they inhibit the growth of normal rat fibroblast (F9 cells) or rat fibrosarcoma (C5 cells) cultures (Table 1).

Suppression is not due to a cytotoxic effect

The SL4c cells were not cytotoxic for various types of target cells: spleen cells, Con A-induced blasts, cells of the EL4 lymphoma line. Nor did SF4c supernatants exert cytotoxic effects (data not shown).

Table 1. SF4c supernatant does not inhibit proliferation of non-lymphoid cells

Cells tested	Dilution of SF4c supernatant	[^{3}H]thymidine incorporation* (c.p.m. ± SE)	% inhibition
Mouse lymphocytes + Con A	No supernatant	26,586 ± 965	
	10^{-1}	4913 ± 36	81·5
	10^{-2}	3997 ± 125	85·0
	10^{-3}	5586 ± 39	79·0
Rat lymphocytes + Con A	No supernatant	47,715 ± 3236	
	10^{-1}	565 ± 20	98·8
	10^{-2}	1026 ± 62	97·9
	10^{-3}	2201 ± 36	95·4
MC11 (mouse fibrosarcoma)	No supernatant	565 ± 35	
	10^{-1}	500 ± 46	11·5
	10^{-2}	570 ± 33	−0·9
	10^{-3}	539 ± 62	4·6
C5 (rat fibroblasts)	No supernatant	2472 ± 432	
	10^{-1}	2592 ± 835	−4·8
	10^{-2}	3237 ± 562	−30·9
	10^{-3}	3656 ± 933	−48·0
F9 (rat fibrosarcoma)	No supernatant	428 ± 35	
	10^{-1}	451 ± 62	−5·3
	10^{-2}	621 ± 111	−45·1
	10^{-3}	509 ± 42	−18·9

* Supernatant from a culture of SL4c cell line was tested for the ability to inhibit proliferation of mouse (B10 strain) or rat (Lewis strain) spleen and lymph node cells as described in the Materials and Methods, and for the ability to inhibit growth of MC11, C5 or F9 cells in a microcytoxicity assay. The values of isotope incorporation obtained in a microcytotoxicity assay are relatively low, but the assay is sensitive enough to detect the suppressive effects since the background in this test is very low (less than 50 c.p.m.) (for details see Holáň, Chutná & Hašek, 1978).

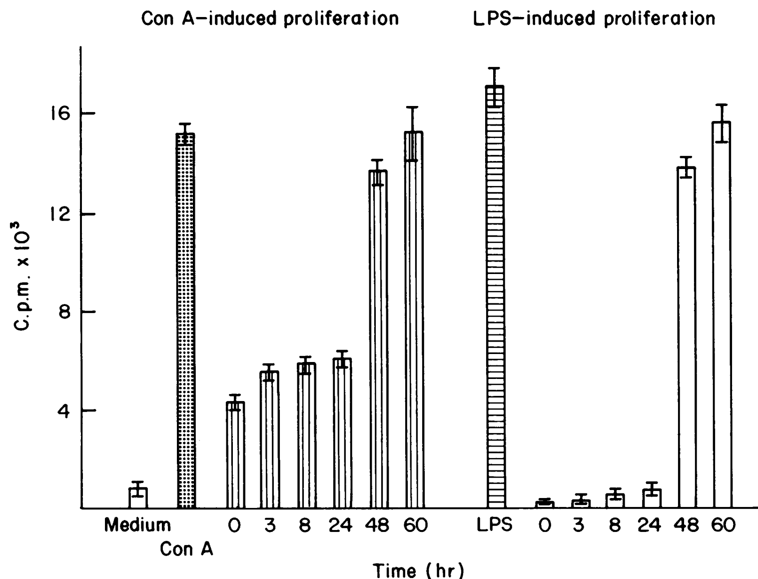

Figure 4. SF4c supernatant inhibits the early stages of cell activation. Spleen/lymph node cells from BALB/c mice were stimulated with Con A or LPS, and SF4c supernatants at a final dilution of $1:10^2$ was added to the cultures at various time intervals after the mitogen. Cell proliferation was assessed by adding [^{3}H]thymidine for the last 8 hr of a 72-hr incubation period.

SF4c supernatant affects the early stages of cell proliferation

SF4c supernatant diluted 1:100 was added to cell cultures at various time intervals after the onset of Con A-induced stimulation. As shown in Fig. 4, the suppressor factor exerted a significant effect on cell proliferation in a 72-hr culture when added in the first 24 hr of cultivation, but it had no effect when added after 48 hr or later.

Phenotype of SL4c cells

Cells of the SL4c line prelabelled with ^{51}Cr were incubated with mAb plus C. The release of radioactivity was high, if anti-Thy 1.2 and/or GK1.5 were used. A higher level of cytolysis than that of the control was also noted with mAb directed against the Lyt 1.2 specificity (Table 2). Lysis with anti-Lyt 1.2 mAb was consistently above the background, but was lower than the effects of anti-Thy 1.2 or Gk1.5 antibodies. The same effects were also observed by trypan blue exclusion in a cytotoxicity test. The expression of Lyt 1 antigen is therefore low, or transient, in only some stages of the cell cycle. One can thus infer that the phenotype of the SL4c line is Lyt $1.2^{\pm}$, Lyt 2.2^{-}, Thy 1.2^{+}, L3T4a^{+}.

Relative molecular weight of the SF4c factor

After fractionation of a 8-ml sample of SF4c-containing supernatant on a Sephacryl S-200 column, all suppressive activity was eluted from the gel at a void volume, which contains compounds with relative MW equal to, or exceeding, 250,000. The fractions yielded during the fractionation of SF4c supernatant on a Sepharose 6B column showed that the MW of the suppressor factor was about 300,000–350,000 (Table 3). Similarly, after fractionation of the SF4c supernatant on an Amicon XM 300 filter, the suppressive activity was found in the fraction characterized by relative MW over 300,000 (data not shown).

Table 2. Phenotype characterization of the SL4c cell line

	% ^{51}Cr release ± SE	
Treatment of SL4c cells	Exp. 1	Exp. 2
–	27·1 ± 2·1	12·1 ± 0·2
C only	24·8 ± 0·6	20·2 ± 2·3
Anti-Lyt 1.2 + C	37·8 ± 1·8	33·7 ± 2·0
Anti-Lyt 2.2 + C	24·0 ± 0·6	24·3 ± 1·6
GK1.5 + C	70·7 ± 3·2	62·6 ± 4·1
Anti-Thy 1.2 + C	64·0 ± 1·6	63·5 ± 3·0

Table 3. Molecular weight of suppressor factor in supernatants from cultures of SL4c cells

Fraction tested*	[^{3}H]thymidine incorporation (c.p.m.) ± SE)	% inhibition
–	38,485 ± 3042	–
1–47		< 10
49	34,922 ± 968	10·1
51	21,920 ± 1716	46·8
53	23,384 ± 1213	42·7
55	17,230 ± 1112	60·0
57	13,544 ± 2623	70·5
59	18,112 ± 24	57·6
61	31,119 ± 475	20·8
63	35,950 ± 1610	7·2
65–79		< 10

* The supernatant from a culture of SL4c cells was fractionated on a Sepharose 6B column, and individual fractions were tested for the ability to inhibit LPS-induced proliferation of BALB/c spleen cells. Background incorporation (cells without LPS) was 3093 c.p.m. Ferritin (450,000), catalase (230,000), albumin dimer (130,000) and albumin monomer (65,000) were used as standards for MW determination.

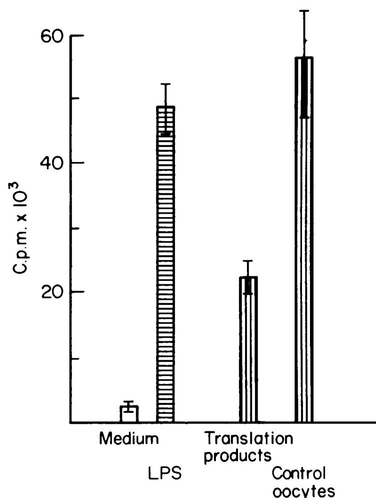

Figure 5. Suppression of cell proliferation by translation products from oocytes injected with RNA isolated from SF4c cells. Twenty μl of incubation medium of the oocytes were added to BALB/c spleen lymph node cells stimulated with LPS, and cell proliferation was assessed by adding [^{3}H]thymidine for the last 16 hr of a 72-hr incubation period.

Translation products of RNA from SF4c cells

Supernatants from cultures of *X. laevis* oocytes injected with RNA from SL4c cells or from cultures of control oocytes were tested for the ability to suppress proliferation of BALB/c spleen/lymph node cells. Supernatants from cultures of the oocytes injected with RNA from SL4c cells inhibited LPS-induced proliferation (Fig. 5).

DISCUSSION

A permanent T-cell line carrying the phenotypic markers of helper/inducer lymphocytes and producing a factor which inhibits proliferation of normal lymphoid cells has been established. Inhibition of B-cell proliferation is much more profound than that of T cells. This is particularly evident if the factor is tested on purified T cells. Inhibition of Con A-induced proliferation of the whole spleen cell population is more profound than in the case of purified T cells. This observation may be due to the suppression of proliferation of non-T cells in the stimulated spleen cell population, and/or the effect of a secondary suppressor factor (factors) of non-T origin that is (are) induced by SF4c factor cannot at present be excluded. Cells of the new line propagate with no requirement for antigenic stimulation and are not cytotoxic for target cells on which they act.

A combination of the Lyt 1^+2^- phenotype with an effector function, manifested as an inhibition of B lymphocyte proliferation, has not yet been recognized. Cells of Lyt 1^+2^- phenotype are often believed to be implicated in the initial steps of suppressor circuits (Germain & Benacerraf, 1981, Green *et al.*, 1983), although Lyt 1^+2^- effector suppressor cells inhibiting T-cell mediated immunity have also been described (Liew, 1983; Holáň & Mitchison, 1984).

In its mechanism of action, namely a direct effect upon B cells, the factor of the cell line described here resembles that produced by a T hybridoma with an inhibitory effect on plaque-forming B cells (Taussig *et al.*, 1979). However, the factor is entirely antigen non-specific, whereas Taussig's factor was found to exhibit an antigen non-specific suppressive effect only at higher concentrations. A common attribute is the high MW of the two factors. For most other suppressor factors, the MW ranges from 35,000 to 70,000, whereas Taussig estimated the relative MW of his factor to approximately 200,000 (Taussig & Holliman, 1979). Recently, a mutant human T-cell line producing

antigen non-specific suppressive factor(s) with an active moiety contained also in the high MW fraction (over 200,000) was described (Lau *et al.*, 1984). In the supernatants from cultured SL4c cells fractionated on a Sepharose 6B column, the suppressor factor was eluted with fractions having relative MW of 300,000–350,000. If the supernatants were divided on an Amicon XM 300 filter, suppressor activity was completely confined to the fraction characterized by a MW over 300,000. However, it is impossible to decide conclusively whether this suppressor factor really has such an unusually large molecule, or whether it is a complex of several smaller subunits, or a smaller molecule bound to a high MW carrier. In order to analyse this question, we are presently testing the activity of the products obtained by *in vitro* translation of RNA fractions isolated from cells of the SL4c line.

The effect of the SL4c cell line is not due to cytotoxicity; neither are the supernatants containing the suppressor factor toxic for lymphoid cells. In this respect, the SL4c line differs from the suppressor line described by Heuer *et al.* (1982), because the latter has the characteristic cytotoxicity activity and the Lyt 1^-2^+ phenotype. The SL4c cells also differ from the Lyt 1^+2^- suppressor line reported by Liew (1983). Liew's line is antigen-specific. Quintáns & Dick (1983) described a soluble factor produced by an antigen-reactive T-cell clone that was lytic for B cells and antibody-producing cells. Their particular lymphotoxin appears to be produced by IL-2-independent T-cell clones but, unlike the SF4c, it also has the capability to inhibit growth of activated B cells, whereas the factor described in this paper suppresses only the activation of B cells and fails to inhibit proliferation of cells that have already been stimulated. Theoretically, the suppressive effect of SF4c supernatants could be due to the harmful effect of membrane fragments from dead cells. In order to avoid this objection, we isolated total RNA from SL4c cells and translated it in *X. laevis* oocytes. The supernatants obtained from oocyte cultures were found to suppress the mitogen-induced proliferation of B cells.

The SL4c line was recloned by single-cell manipulation (data not shown). All of the six clones obtained expressed the same Lyt $1.2^{\pm}$, L3T4a$^+$, Lyt 2.2^-, Thy 1.2^+ phenotypic markers and produced a non-specific suppressor factor. This suggests that long-term propagation of these cells resulted in selection of a single cell type, or that cells with the described characteristics prevailed in the cell line.

We do not know what are the favourable conditions of activation of the cell lines with suppressive effects. However, another two cell lines of BALB/c or B10.A origin established under the conditions described here showed a similar suppressive effect. In some aspects, the conditions for growing the SL4c cell line resemble those described for cloning of natural suppressor cells by Oseroff, Okada & Strober (1984).

Because of the, as yet unreported, combination of helper/inducer phenotype with a non-specific suppressive effect acting directly upon the early stages of cell activation, the described SL4c line represents another hitherto unrecognized cell type involved in the suppressor systems. We have observed that administration of the SF4c factor *in vivo* inhibits allotransplantation reactions (Hašek, Holáň & Vančatová, 1985) and prevents induction of experimental allergic encephalomyelitis in rats (J. Štědra *et al.*, manuscript in preparation). The exact biological role of these cells is presently being investigated in more detail.

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