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# THYMUS DERIVED INHIBITOR OF LYMPHOCYTE PROLIFERATION

## III—PARTIAL PURIFICATION AND CHARACTERISTIC BIOLOGICAL ACTIVITY

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**Abstract**—A crude factor isolated from bovine thymus was partially purified using ion exchange chromatography. The resulting fraction was tested for inhibitory properties towards the proliferation of various human and murine lymphoid cells in culture. It was found that this partially purified thymic factor (TF) strongly inhibited the proliferation of lymphocytes induced by mitogen, antigen or allogeneic stimuli with minimal effects on spontaneous proliferation. The inhibition was partially reversible and at the level of the lymphocyte rather than through mediation by non-lymphoid cells. Data obtained in kinetic studies suggest that TF interferes with an early event in the stimulation process, resulting in delayed lymphocyte proliferation.

Numerous factors influencing lymphoid cell growth have been reported (Waksman & Namba, 1976), some of which are thought to be lymphoid chalones (Rytömaa, 1978). The chalones isolated from various lymphoid tissues have general inhibitory effects on lymphoid cell proliferation being cell-specific but not species-specific (Bullough & Rytömaa, 1965). We described previously a thymus crude factor (TCF) isolated from bovine thymus tissue which interfered with lymphoid DNA synthesis *in vitro* as well as *in vivo* (Rijke, Lempers & Ballieux, 1978; Rijke & Ballieux, 1979). Furthermore, it has also been demonstrated that TCF is not related to, or contaminated with, polyamines (Rijke & Ballieux, 1978). The data obtained so far indicated the presence of a "chalone-like" inhibitory moiety in our TCF. In the present study attempts were made to obtain more purified TCF preparations, in order to analyse more precisely the mechanism of the inhibitory activity. Several reports have been published on the purification of lymphocyte inhibiting factors from crude lymphoid tissue extracts. Most investigators used gel filtration to fractionate crude inhibitor preparations (Lenfant, Privat de Garilhe, Garcia-Giralt & Tempête, 1976; Hiestand, Borel, Bauer, Kis, Magnée & Stähelin, 1977; Blazsek & Gaál, 1978) or combinations of ultrafiltration, ion exchange chromatography and gel filtration (Kiger, Florentin, Lorans & Mathé, 1977). Recently, it was suggested that this approach may be inadequate since it was found that lymphocyte chalone fractions were bound to RNA and would therefore be eluted as complex(es) (Houck, Kanagalingam, Hunt, Attalah & Chung, 1977). To circumvent this problem, we decided to adopt a purification procedure using ion-exchange chromatography (Gianfranceschi, Amici & Guglielmi, 1975). The resulting partially purified thymic factors (TF) were studied for their ability to suppress lymphoid cell DNA synthesis and attempts were made to determine the putative working pattern of this TF preparation.

## EXPERIMENTAL PROCEDURES

### *Preparation of the extracts*

Thymus crude factor (TCF) was prepared from fresh bovine thymus tissue as described previously (Rijke & Ballieux, 1979).

### *Purification of the extracts*

The method originally described by Gianfranceschi *et al.* (1975) was used with slight modification. In brief, 1000 mg of TCF was dissolved in 100 ml of 0.005 M ammonium acetate/acetic acid buffer pH 5.0 and chromatographed on a DEAE cellulose (DE52, Whatman) column (2.8 × 46 cm) equilibrated with the same buffer. After the unabsorbed proteins were eluted, the column was washed with this buffer until effluent absorbance values at 280 nm reached baseline. The unabsorbed fractions were lyophilized and dissolved in 25 ml distilled water and chromatographed on a DOWEX 50WX2 (Fluka, Buchs, Switzerland) column (2.8 × 46 cm) equilibrated with 0.005 M ammonium acetate/acetic acid buffer at pH 4.5. After the unabsorbed proteins were eluted with the same buffer, the column was washed with the buffer until baseline OD280 values were reached. Fractions were subsequently eluted stepwise with,

0.08 M ammonium acetate/acetic acid buffer, pH 6.0; 0.2 M ammonium acetate/ammonia, pH 9.5; and finally with 1.0 M ammonium acetate/ ammonia, pH 9.5. Resulting protein fractions were pooled and the pH adjusted to 7.0 before lyophilization. The protein content of the fractions was determined using the method of Lowry, Rosebrough, Farr & Randall (1951).

*Animals*

Swiss and occasionally BALB/c mice were used throughout the experiments. Mice were reared under standard conditions, housed in plastic cages and provided with food pellets and water *ad libitum*.

*Cell cultures*

*Induced lymphocyte proliferation.* For human lymphocytes the method as described previously was used (Rijke & Ballieux, 1979). In addition to stimulation with mitogens or allogeneic cells, lymphocytes were stimulated with the following antigens: 66 μg *Candida* sp. (Allergenen Lab., Haarlem, the Netherlands), 13.3 μg purified protein derivative (PPD) (Statens, Copenhagen, Denmark), 3.3 LF tetanus toxoid (Statens, Copenhagen, Denmark) per well respectively. Cultures were incubated in the presence or absence of Millipore-sterilized fractions for 6 days.

Mouse lymphocytes were obtained in the following fashion. Six-week-old female mice were killed by cervical dislocation. Spleens, thymuses and lymph nodes (axillary and inguinal) were aseptically removed and cut into pieces with surgical knives in a few mls of Tris buffered minimum essential medium (MEM). The tissue fragments thus obtained were extensively washed with culture medium. The pieces were allowed to sediment and the cell-rich supernatant was centrifuged for 10 min at 200 **g**. The cells were resuspended at a concentration of $6 \times 10^6$ lymphocytes per ml of culture medium, consisting of buffered (24 mM $NaHCO_3$ and 25 mM HEPES) RPMI 1640 supplemented with penicillin (100 I.U./ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and pooled human AB serum (10% v/v). Of this suspension samples of 0.05 ml per well representing $3 \times 10^5$ cells were cultured in microtiter plates (Dynateck, M24AR, Greiner, Nürnberg, GDR) in the presence of either 0.05 ml mitogen solution or 0.05 ml of a BALB/c lymphocyte suspension ($6 \times 10^6$ ml) in the case of the mixed lymphocyte reaction (MLR) plus 0.05 ml of the sterile fraction to be tested. In pilot studies optimal proliferative responses were found when 0.5 μg concanavalin A (Con A) (Miles-Yeda, Elkhart, U.S.A.), 10 μg phyto-haemagglutinin (PHA) (Wellcome, Beckenham, U.K.), 10 μg *E. coli* 0127: B8 lipopolysaccharide (LPS) (Difco, Detroit, U.S.A.) or 20 μg pokeweed mitogen (PWM) (Gibco, New York, U.S.A.) per well were used. Cultures were incubated at 37°C in an atmosphere of 5% $CO_2$ in air for 48 h. Seven hours before termination of the cultures 1 μCi (methyl-$^3$H)thymidine ($^3$H-Tdr, 5 Ci/mM), 1μCi (5,6-$^3$H) uridine ($^3$H-UR, 45 Ci/mM) or 1 μCi L-(4, 5-$^3$H) leucine ($^3$H-Leu, 40 Ci/mM) (Radiochemical Centre, Amersham, U.K.) was added to the suspension in each well. Incorporation of radioactivity was assessed as described (Rijke & Ballieux, 1979) using an automatic harvester (Skatron, Lierbyen, Norway).

*Morphology*

Morphological examination of the cells after culturing was done after fixation of the cells using sodium citrate and methanol/acetic acid according to a procedure described previously (Rijke & Ballieux, 1979).

*Spontaneous proliferation*

The method used has been described earlier (Rijke & Ballieux, 1979). In brief, the following cells were used to set up the cultures:

- Mouse thymocytes: these cells were isolated from aseptically removed Swiss mouse thymuses following an established procedure (Rijke & Ballieux, 1979).
- Mouse leukemia cells: derived from an established lymphoblastic leukemia (L1210).
- Mouse mastocytoma cells: derived from a continuous cell line, P815 × 2, syngeneic to DBA/2.
- Human leukemic T cells: derived from a T lymphoblastoid cell line (CCRF-CEM).
- Human leukemic B cells: derived from a B lymphoblastoid cell line (NC37).

Cell lines were kindly provided by Drs. Zeijlemaker (Amsterdam, the Netherlands)(P815) and A. v. d. Linde (Utrecht, the Netherlands) (L1210, CCRF-CEM, NC 37). The cells were grown as suspension cultures in RPMI 1640 supplemented with fetal calf serum (10% v/v), antibiotics and L-glutamine. Before use in the culture systems, the cells were extensively washed with medium.

*Cell counts and viability*

At the end of each culture period, cell counts were made using a hemocytometer and cell viability was assessed using the trypan blue exclusion test.

*Calcium uptake assay*

The uptake of $^{45}$Ca by lymphocytes was determined by the method originally described by Whitney & Sutherland (1973). In brief, $10^7$ mouse spleen cells

were prewarmed at 37°C in 600 μl RPMI 1640 without bicarbonate, and 1 μCi $^{45}Ca$ ($^{45}CaCl_2$, 42 Ci/g, Radiochemical Centre, Amersham, U.K.) was added together with the agent to be tested, reaching a final volume of 900 μl. After incubation for 5 min at 37°C in a water bath, the reaction was stopped by adding cold RPMI to the cells. The cells were then washed three times and transferred onto Millipore filters (pore size 5 μm). Filters were solubilized in dioxane and scintillation fluid (MI96, Packard, Groningen, the Netherlands) was added. Radioactivity was determined by liquid scintillation counting (Searle, Mark III).

*Sensitivity of TF to various treatments*

To investigate the sensitivity of TF to heat-treatment, different aliquots of TF (150 μg/100 μl MEM) were incubated in glass tubes for 10 min at 56°C and 100°C. After the incubation period the tubes were placed in ice, and the supernatant was recovered, after the coagulated material had been spun down in the cold. The sensitivity of TF to other treatments was studied by treating TF (150 μg/100 μl phosphate buffered saline, PBS) with 10 μl (30 μg) trypsin (Fluka, Buchs, Switzerland), 10 μl (30 μg) DNAase (Worthington, Freehold, U.S.A.), 10 μl (30 μg) pronase (Merck, Darmstadt, G.D.R.) or 10 μl (1.5 mM) 2-mercaptoethanol (2-ME) for 2 h or 20 h. At the end of the incubation period, trypsin treatment was stopped by adding 10 μl (30 μg) trypsin inhibitor from soy bean (Fluka, Buchs, Switzerland). The sensitivity of TF to acid and alkali was studied by incubating TF (150 μg/100 μl PBS) with sodium hydroxide or acetic acid (0.5 M final concentration) for 2 h at 37°C. After this period the samples were neutralized. The aliquots were tested for inhibitory properties, with similarly treated samples of PBS serving as control.

## RESULTS

Four thymus fractions isolated from four different thymuses were purified by DEAE cellulose and DOWEX column chromatography, resulting in a partially purified thymic factor (TF). As seen in Table 1, this purification procedure leads to increased specific inhibitory activity of the eluted fractions.

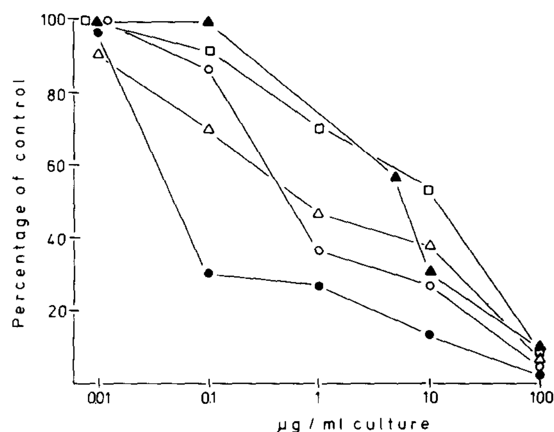


Fig. 1. Typical effect of thymic factor (TF) on the proliferation of human peripheral blood lymphocytes, expressed as the percentage of DNA synthesis ($^3H$-thymidine incorporation) as compared to values (control) obtained using allogeneic lymphocytes (●), PHA (○), Con A (Δ), PWM (□) and *Candida* sp. (▲). The variation of the mean of quadruplicate cultures in the corresponding experiments was generally 10–15%.

*Effect of TF on induced proliferation*

TF inhibited the proliferation of human peripheral lymphocytes as induced by mitogens, antigen or allogeneic cells (Fig. 1.). Similar results were obtained when mouse spleen cells were stimulated with mitogens or allogeneic cells (Fig. 2.). The suppression of DNA synthesis was dose-dependent. Both DNA and RNA synthesis was inhibited: protein synthesis was inhibited to a lesser extent (Fig. 3). The inhibitory activity of a given amount of TF seemed to be somewhat dependent on the total number of cells used (Fig. 4). When TF was added to mitogen-stimulated cultures of lymphoid cells isolated from other lymphoid organs, it was found that thymus and

Table 1. Ion exchange chromatography of a thymus crude factor batch

| Fraction | Total protein (mg) | Total activity (U) | $ID_{50}$* | Yield (%) |
|---|---|---|---|---|
| Crude fraction | 1000 | 4000 | 250 | 100 |
| DEAE cellulose (DE52)† | 100 | 2000 | 50 | 50 |
| DOWEX 50WX2 (1.0 M)† | 8.8 | 1760 | 5 | 44 |

* Inhibitory dose (μg/ml) to obtain 50% suppression of the DNA synthetic response of mouse spleen cells stimulated with LPS.

† Other eluted peaks did not exhibit inhibitory properties at doses below 100 μg/ml.

spleen cells were inhibited to an equal extent while lymph node cells were less susceptible to the inhibitory activity of TF (Fig. 5). Furthermore, the inhibitory activity of TF was not based on cytotoxic properties as can be seen from Fig. 6; the number of viable cells in the TF-treated cultures did not decrease below that of non-stimulated cells. Moreover, in experiments using human lymphocytes it was found that the inhibition of blast transformation, as judged from the morphology in cell smears, correlated with the inhibition of $^3$H-thymidine uptake (Table 2).

*Effect of TF on spontaneous proliferation*

In our previous papers we described the inhibition by TCF of spontaneously proliferating lymphocytes in short term cultures (Rijke & Ballieux, 1978, 1979). Therefore, TF was tested for inhibitory properties on various cultured cells which were proliferating without stimulation. As shown in Fig. 7, only when relatively high concentrations of TF were used was a suppression of DNA synthesis found. The inhibition at these doses, however, was not strictly lymphocyte specific since mastocytoma cells were also affected.

*Some aspects of the working pattern of TF*

*Reversibility.* The inhibitory effect of TF on mouse spleen cells was reversible when spleen cells were incubated for 6 h with TF, then washed and stimulated with the appropriate mitogen. However, upon incubation for longer periods (24 h) it was found that lymphocytes pre-incubated with TF did not respond as well to a mitogenic stimulus compared to the medium treated cells (Table 3).

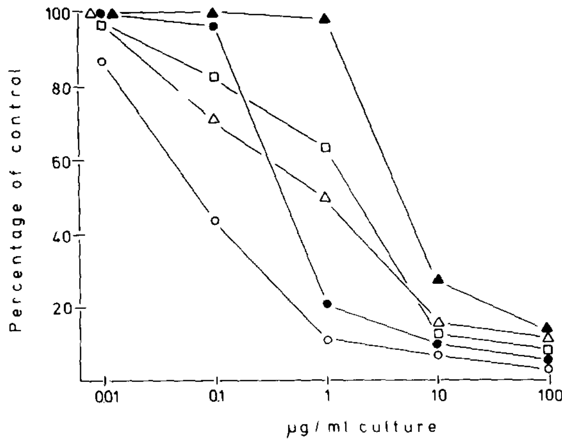


Fig. 2. Typical effect of TF on the proliferation of mouse spleen cells, expressed as the percentage of DNA synthesis ($^3$H-thymidine incorporation) as compared to control values, as induced with allogeneic lymphocytes (●), LPS (○), PHA (Δ), Con A (▲) and PWM (□). The variation of the mean of quadruplicate cultures in the corresponding experiments was generally 10–15%.

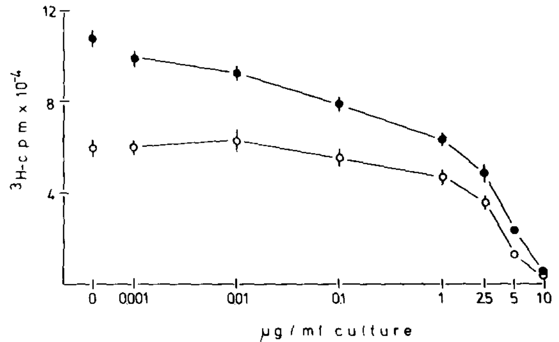


Fig. 3. Effect of TF on RNA and protein synthesis of LPS stimulated mouse spleen cells, (●) $^3$H-U R incorporation, (○) $^3$H-Leu incorporation. Each point represents the mean of quadruplicate cultures expressed in counts per minute (cpm) with corresponding standard deviation (S.D.). A representative experiment is shown.

Table 2. Effect of thymic factor (TF) on blast transformation and thymidine incorporation in human two-way mixed lymphocyte cultures

| Lymphocytes | Test conditions | Viable lymph. × $10^5$/ml | % Blasts | % Inhibition Blasts | % Inhibition ($^3$H) TdR |
|---|---|---|---|---|---|
| Donor A | medium | 2.6±0.2* | — | — | — |
| Donor B | medium | 2.5±0.2 | — | — | — |
| Donor A + B | medium | 11.7±0.6 | 79±8 | 0 | 0 |
| Donor A + B | TF† | 5.6±1.3 | 16±4 | 80±2 | 89±7 |

* Data expressed as the mean of three experiments ±S.E.M.
† 15 μg/ml.

Table 3. Partial reversibility of the action of TF after preincubation*

| Preincubation (h) | Cells pre-incubated in | Stimulation afterwards | | | | |
|---|---|---|---|---|---|---|
| | | LPS | PWM | PHA | Con A | none |
| 6 | Medium | 10276±1373† | 9777±1507 | 128534±11581 | 23059±2651 | nt |
| | TF§ | 18164±734 (0)‡ | 9556±542 (3) | 149700±11616 (0) | 9825±680 (58) | nt |
| 24 | Medium | 34243±3903 | nt‖ | nt | 106968±8044 | 1157±340 |
| | TF | 19127±1837 (44) | nt | nt | 28297±3586 (74) | 629±114 |

* Data are shown from a representative (out of five) experiment.
† DNA synthesis expressed in cpm±standard deviation (S.D.).
‡ Percentage inhibition as compared to control values.
§ 10 μg/ml.
‖ Not tested.

*Late addition.* When TF was added to cultures of mitogen-stimulated spleen cells at different times after the start of the cultures it was found that TF was only fully active when added within 12–24 h (Fig. 8). Similar results were obtained in stimulation tests using human lymphocytes (data not shown).

*Lack of interference with mitogen.* The inhibitory activity of TF could be based on the binding or complexation of mitogen, leading to a decrease of mitogen present. This effect should then be overcome by the addition of increasing amounts of mitogen. As seen in Fig. 9 however, TF was capable of suppressing the DNA synthetic response of mitogen-stimulated mouse lymphocytes regardless of the concentration of mitogen used for the stimulation process.

*Kinetics of the TF-mediated inhibition.* The effect of TF on early DNA, RNA and protein synthesis was

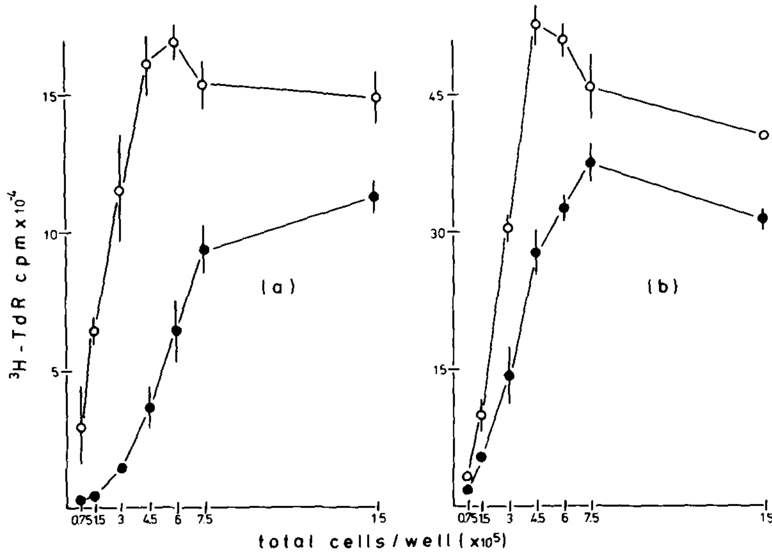


Fig. 4. Influence of the number of cells used on the suppressive activity of TF on mouse spleen cell proliferation, DNA synthesis, $^3$H-thymidine incorporation expressed in cpm as induced with (a) LPS or (b) Con A, (○) Medium control, (●) TF (10 μg/ml). Each point represents the mean of quadruplicate cultures with corresponding S.D. A representative experiment is shown.

studied in kinetic experiments using Con A- or LPS-stimulated mouse spleen cells. These cells were cultured for various periods of time and subsequently pulsed with $^3$H-TdR, $^3$H-UR or $^3$H-Leu for 2 h. As shown in Fig. 10, the rate of $^3$H-UR and $^3$H-Leu incorporation in Con A-stimulated cells exceeded that of non-stimulated cells at 6–12 h and continued to increase throughout the first 40–60 h of culture. DNA synthesis significantly rises at 12–24 h of culture, reaching an optimum around 48–60 h. When TF was present throughout the culture period, DNA and protein synthesis were inhibited over the period 12–60 h from the start of the cultures. After this time lapse, DNA synthesis in the presence of TF surpasses that obtained in cultures containing medium only. In

Table 4. Effect of TF on early calcium uptake of mouse spleen cells*

| Agent | $^{45}$Ca uptake |
|---|---|
| Medium | 707±62† |
| Con A (1 μg/ml) | 740±38 |
| Con A (10 μg/ml) | 829±10 |
| TF (10 μg/ml) | 647±41 |

* Data are from a representative experiment out of three.
† Mean cpm of triplicate cultures±S. D.

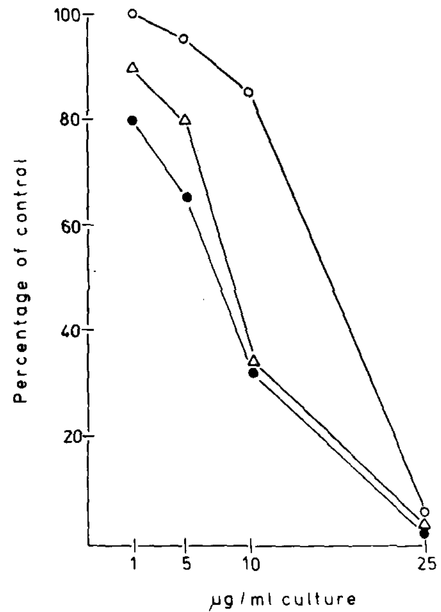


Fig. 5. Sensitivity of various mouse lymphoid cells for the inhibitory activity of TF, expressed as the percentage of DNA synthesis ($^3$H-thymidine incorporation) as compared to control values of PHA stimulated cultures of spleen cells (●), thymocytes (Δ) and lymph node cells (○). The variation of the mean of quadruplicate cultures in the corresponding experiments was generally 10—15%. A representative (out of three) experiment is shown.

Table 5. Sensitivity of TF to various treatments. Measured on LPS stimulated mouse spleen cells*

| Treatment | Untreated | | Treated | |
|---|---|---|---|---|
| | Control | TF† | Control | TF |
| Trypsin | 62076± 7900‡ | 1100± 482 (98)§ | 64515± 2367 | 12467± 793 (81) |
| Trypsin (20 h) | 152703±16282 | 19837±7219 (87) | 86672± 7690 | 23219±5755 (73) |
| Pronase | 60642± 3422 | 10732± 907 (82) | 48980± 3242 | 8955±1738 (82) |
| Pronase (20 h) | 152703±16282 | 19837±7219 (87) | 111900±16324 | 47690±9441 (58) |
| DNase | 62076± 7900 | 1100± 482 (98) | 23982± 2977 | 3074± 855 (87) |
| 2 ME | 116624±22505 | 13728±1036 (88) | 80807±12678 | 34072±9967 (58) |
| Acid | 41349± 2631 | 2363± 784 (96) | 38656± 6940 | 6021±1260 (84) |
| Alkaline | 41349± 2631 | 2363± 784 (96) | 22892± 3694 | 9597±1583 (59) |
| 10 min 56°C | 32650± 1914 | 419± 114 (99) | — | 145± 142 (100) |
| 10 min 100°C | 32650± 1914 | 419± 114 (99) | — | 1767± 223 (84) |

* Data from a representative experiment are shown.
† 10 μg/ml.
‡ DNA synthesis, $^3$H-thymidine incorporation expressed in cpm±S.D.
§ Percentage inhibition as compared to control values.

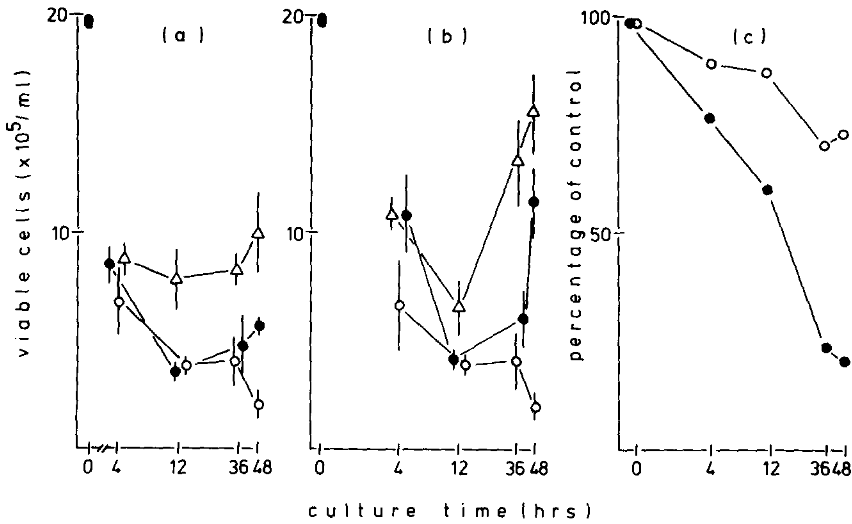


Fig. 6. Effect of TF on viable cells and proliferation of mitogen-stimulated mouse spleen cells as induced with (a) LPS and (b) Con A, (Δ) mitogen, (●) mitogen plus TF (10 μg/ml), (○) unstimulated. Each point represents the mean of triplicate cultures with corresponding S.D. The percentage of DNA synthesis induced by TF as compared to control values is given in (c) of LPS (●) and Con A (○) stimulated cultures. A representative (out of two) experiment is shown.

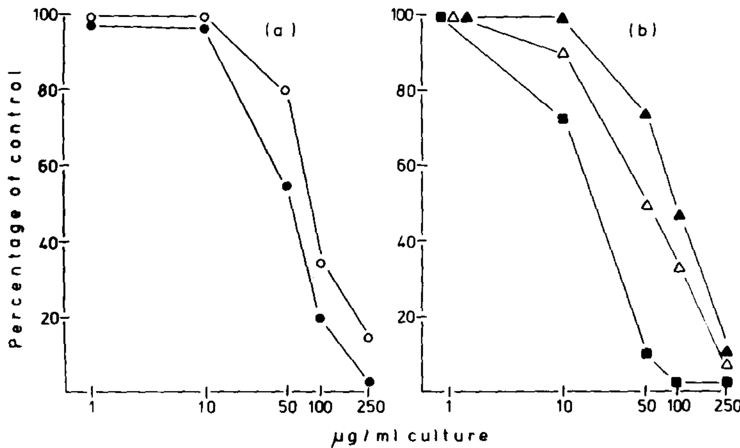


Fig. 7. Typical effect of TF on spontaneous cell proliferation, expressed as the percentage of DNA synthesis ($^3$H-thymidine incorporation) as compared to control values of (a) human leukemic lymphocytes, (●) NC37 and (○) CCRF-CEM, (b) mouse cells, (■) L1210, (Δ) thymocytes, (▲) mastocytoma P815. The variation of the mean of quadruplicate cultures in the corresponding experiments was generally 10–15%. A representative (out of three) experiment is shown.

contrast, RNA synthesis was only slightly enhanced relative to medium-containing cultures during the first 30 h of culture. It then remains relatively constant for the next period between 40 and 48 h after which a further increase of RNA synthesis occurs. In experiments using LPS-stimulated cells, DNA, protein and to a lesser extent RNA synthesis was blocked by TF during the entire culture period (Fig. 11). Moreover, at a lower concentration of TF (2 μg/ml) the same limited inhibition pattern was

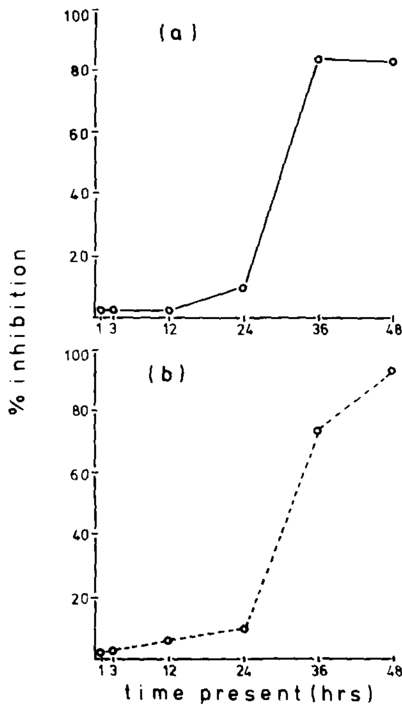


Fig. 8. Typical effect of late addition on the suppressive activity of TF (10 μg/ml) on DNA synthesis of mouse spleen cells, expressed as percentage inhibition of DNA synthesis ($^3$H-thymidine incorporation) as compared to control values, stimulated with (a) (O—O) LPS and (b) (O··O) Con A. The variation of the mean of quadruplicate cultures in the corresponding experiments was generally 10–15%.

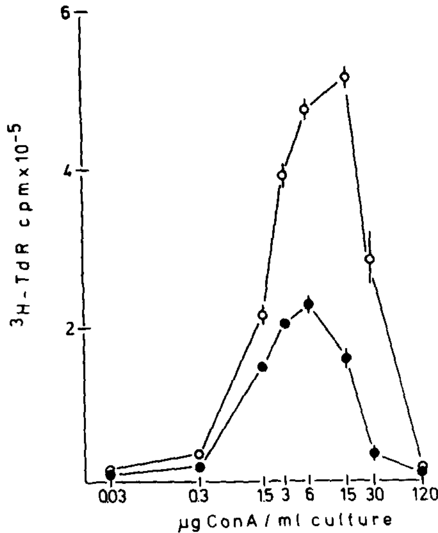


Fig. 9. Influence of various concentrations of Con A on the suppressive activity of a given amount TF, DNA synthesis, $^3$H-thymidine incorporation expressed in cpm, of mouse spleen cell proliferation. (O) culture medium; (●) TF (10μg/ml). Each point represents the mean of quadruplicate cultures with corresponding S.D. A representative experiment is shown.

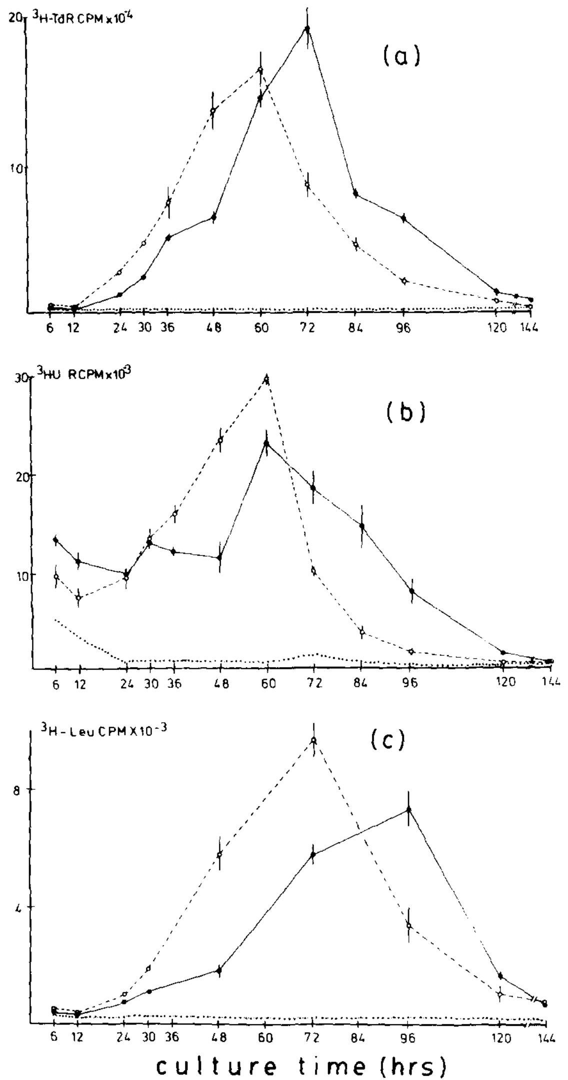


Fig. 10. Effect of TF on Con A-stimulated mouse spleen cell proliferation. (a) DNA synthesis, (b) RNA synthesis, (c) protein synthesis. Each point represents the mean of quadruplicate cultures expressed in cpm with corresponding S.D. (O) Con A, (●) Con A plus TF (10 μg/ml), (···) unstimulated. A representative experiment (out to six) is shown.

found as observed in Con A-stimulated cells (data not shown).

*Effect of TF on calcium uptake.* As shown in Table 4, TF did not significantly affect the early uptake of calcium, in contrast to Con A which was found to cause an increase in calcium uptake.

*Sensitivity of TF to various treatments.* The effect of various treatments on the inhibitory activity of TF is summarized in Table 5. The suppressive activity of TF is heat-stable and is not diminished by DNAase, trypsin or acid treatment. However, when TF is treated with 2-ME, alkali or pronase for 20 h, the inhibitory activity is reduced.

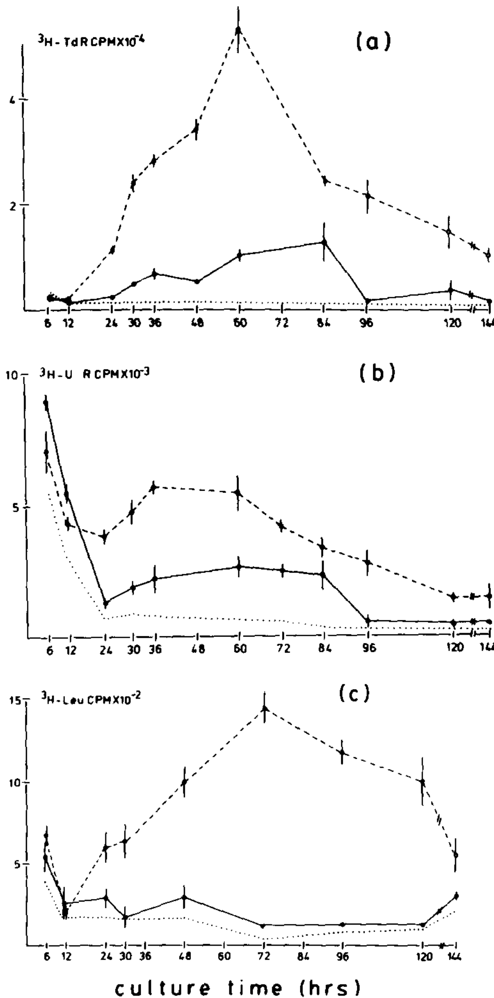


Fig. 11. Effect of TF on LPS stimulated mouse spleen cell proliferation. (a) DNA synthesis, (b) RNA synthesis, (c) protein synthesis. Each point represents the mean of quadruplicate cultures expressed in cpm with corresponding S.D. (○) LPS, (●) LPS plus TF (10 μg/ml), (···) unstimulated. A representative experiment (out of six) is shown.

## DISCUSSION

In previous reports we demonstrated that a thymus crude factor (TCF) inhibited human lymphocyte DNA synthesis *in vitro* (Rijke & Ballieux, 1979) as well as murine lymphocyte DNA synthesis *in vitro* (Rijke & Ballieux, 1978) and *in vivo* (Rijke, Lempers & Ballieux, 1980). It has been reported that crude extracts from lymphoid tissue contain several unidentified, nonspecific suppressive factors (Hiestand *et al.*, 1977; Blazsek & Gaál, 1978) as well as compounds like thymidine (Kasahara & Shiori-Nakano, 1976; Lenfant, Garcia-Giralt, Thomas & di Giusto, 1978) and the polyamines (Allen, Smith, Curry & Gaugas, 1977). Since these factors could interfere with the test systems used in our study, TCF was further purified by ion exchange chromatography. This procedure resulted in the isolation of a relatively active thymus factor (TF) from the corresponding crude factor (TCF).

Low amounts of TF suppressed the proliferation of human and murine lymphocytes as induced by stimulation with mitogens or allogeneic cells. It is unlikely that this suppressive activity is caused by interference of TF with the mitogen used, since no shift in the dose response profile of Con A to higher concentrations was observed by the addition of TF (Fig. 9). Moreover, TF was still able to suppress lymphocyte proliferation when added 12–24 h after the initial mitogenic stimulation at the start of the cultures (Fig. 8). Furthermore, cell proliferation induced by stimulation with allogeneic lymphocytes was equally well inhibited (Fig. 1 and 2); the latter stimulation process involving mere cell–cell interaction (Bach, Bach & Sondel, 1976).

It follows from the late addition experiments and from the data on the kinetics of TF-mediated inhibition that TF interferes with cellular events occurring shortly after interaction with mitogen or allogeneic cells (Decker & Marchalonis, 1978) rather than affecting cells already actively synthesizing DNA (Fig. 10a and 11a). This could explain our present finding that TF, in contrast to TCF (Rijke & Ballieux, 1978, 1979) fails to suppress the DNA synthesis of spontaneously proliferating cells.

In kinetic experiments shown in Fig. 10, TF suppressed DNA, protein and to a lesser extent, RNA synthesis in Con A-stimulated cells during a restricted period only. The inhibition can be overcome by extending the culture period. In contrast, the LPS-induced proliferation was completely suppressed throughout the entire culture period (Fig. 11). Only when lower concentrations of TF were added to the cells was an inhibition profile similar to that obtained in Con A-stimulated cultures observed. These results

seem to indicate that T-cells (stimulated by Con A) are less susceptible than B-cells (stimulated by LPS) (see also Fig. 2); this observation merits further investigation using purified T- and B-cell populations. Furthermore, it was noticed that lymph node cells were less susceptible to the inhibitory activity of TF than were spleen cells or thymocytes. This could be due to differences in the cellular composition of these organs in terms of T-B-cell ratios or differences in macrophage contents. At this point, it should be noted that TF probably does not mediate its inhibitory activity through macrophages, since mitogen-stimulated spleen cells depleted of phagocytic cells could still be suppressed by TF (Rijke *et al.*, unpublished observations).

The precise action of TF during the early period of mitogen-stimulated cultures remains unclear. We could show (Table 4) that TF did not interfere with the early uptake of calcium, a substance regarded as a second messenger in mitogen-stimulation (Wedner & Parker, 1976; Decker & Marchalonis, 1978). It has been reported that a rise in intracellular cyclic AMP may lead to inhibition of DNA synthesis (Wedner & Parker, 1976). Indeed, the inhibitory activity of a number of lymphoid "chalone-like" factors may be mediated by a rise in intracellular levels of cyclic AMP (Attallah & Houck, 1977; Jegasothy, Namba & Waksman, 1978). Our efforts to demonstrate a significant rise in cyclic AMP levels in mouse lymphocytes were unsuccessful. Some factors which have recently been described inhibit lymphoid cell proliferation through the inhibition of DNA-directed polymerases (Gianfranceschi *et al.*, 1975; Amici, Rossi, Cioé, Matarese, Dolei, Guglielmi & Gianfranceschi, 1977). We have not yet investigated whether our TCF has a similar activity, but it should be stressed that TF does not interfere with spontaneously proliferating cells, Moreover, we assume that TF is different from the factors reported by others. This assumption is supported by the fact that the inhibitory activity of both TF and TCF, in contrast to the factors mentioned above, can be reduced by pronase, 2-ME or alkaline treatment (Rijke *et al.*,1980). Furthermore, preliminary results strongly suggest an active factor of relatively small molecular weight to be present in TF (Rijke *et al.*,1978), thus distinguishing TF, on these grounds alone, from other "chalone-like" factors.

The importance of cell-derived factors in the physiological control of lymphocyte proliferation is increasingly recognized (Waksman & Namba, 1976; Waksman & Wagshal, 1978). The production and secretion of these factors by lymphocytes has not always been demonstrated. Only the inhibitor of DNA synthesis (IDS) (Namba, Jegasothy & Waksman, 1977; Lee & Lucas, 1977) and the soluble immune response suppressor (SIRS) (Tadakuma, Kühner, Rich, David & Pierce, 1976) have been reported to be produced by lymphocytes. However, it was recently shown that SIRS acts on macrophages (Tadakuma & Pierce, 1976) and that IDS also inhibits the proliferation of non-lymphoid cells (Lee & Lucas, 1978; Wagshal, Jegasothy and Waksman, 1978). These factors therefore cannot be considered as "classical" lymphocyte chalones. It is difficult to state that TF should be regarded as a lymphocyte chalone since, according to the classical definition (Bullough & Rytömaa, 1965), a chalone should inhibit lymphocyte proliferation regardless of whether it occurs spontaneously or is induced. Therefore, one could argue that TF bears more resemblance to a cytokine (Waksman & Wagshal, 1978) affecting only induced lymphocyte proliferation. The biological activity of TF resembles, at least to a certain extent, that of Cyclosporin A, a potent immunosuppressive drug which strongly inhibits induced lymphocyte proliferation (White, Plumb, Pawelec & Brons, 1979).

From the data available in the literature at present (Cooperband, Nimberg, Schmid & Mannick, 1976; Waksman & Wagshal, 1978; Rytömaa, 1978) it can be concluded that several, obviously different inhibitors of lymphocyte proliferation exist. Only after intensive purification and careful assessment of the biological activity can the identity of these factors be established with certainty.

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