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Immunology

Induction of T-cell maturation by a cloned line of thymic epithelium (TEPI)

(interleukin 2 production/cytolytic T-cell activity)

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ABSTRACT **A cloned cell line of thymic origin has been characterized as epithelial in nature. A description of the procedures for derivation and cloning of the cell line includes use of epidermal growth factor. The thymic epithelial (TEPI) cell line is Ia antigen positive, forms desmosomes, and produces an extracellular fibronectin matrix. The supernatant from confluent monolayers of TEPI was tested for its ability to promote thymocyte functional activity. TEPI supernatant (TEPI SN) was demonstrated to greatly enhance the response of peanut agglutinin-positive thymocytes to alloantigen, as measured by cell-mediated lympholysis. Furthermore, preincubation of peanut agglutinin-positive thymocytes with TEPI SN prior to allostimulation resulted in marked enhancement, thus distinguishing it from interleukin 2. Finally, TEPI SN was demonstrated to induce interleukin 2 production by peanut agglutinin-positive thymocytes in the presence of concanavalin A. This activity was demonstrated not to be due to interleukin 1, which is absent in TEPI SN. Preliminary biochemical analysis indicates that the biological activity is associated with a M_r 50,000 entity. The data suggest that TEPI produces a soluble factor capable of inducing function of an immature thymocyte subpopulation into an IL 2 producer.**

It is now well established that the thymic stromal microenvironment is an essential determinant in the proper development of bone marrow progenitors committed to the T-cell lineage into thymocytes capable of emigrating to the peripheral lymphoid tissues as functional T lymphocytes (1–5). The thymic stroma is composed of at least three components: fibroblastic, epithelial, and macrophage (6), all of which may be involved in the differential events in the thymus. In fact, it has been reported that primary explants of thymic stromal tissue are capable of effecting the maturation of thymocytes to mixed lymphocyte reaction (MLR)- and phytohemagglutinin-reactive lymphocytes (7). Later studies have reported that "epithelial" monolayers are capable of causing maturation of spleen cells from immunodeficient mice (8, 9). However, the problem with such studies is the predominance of macrophages and other nonepithelial cell types in primary explants of thymic stroma. Because macrophages have also been reported to secrete a factor that is important in at least one stage of thymic maturation (10), it is difficult to assign to epithelial cells the maturation phenomena reported with primary explants. More recently, it has been reported that a specialized "epithelial-like" cell is capable of engulfing thymocytes and processing them (11).

The most definitive studies have been carried out with supernatants from primary explants of rat thymic epithelium. It has been demonstrated that the culture supernatant from these cells increases the cAMP level of thymocytes, converts phytohemagglutinin- and MLR-nonreactive thymocytes to responsive lymphocytes, and acts upon the immature peanut agglutinin-positive (PNA^+) population of thymocytes (12). Furthermore, the maturation-inducing activity of this supernatant was distinct from the substitution for helper T cells provided by interleukin 2 (IL 2).

A variety of soluble mediators of thymic origin has been reported to affect cell surface antigen expression, enhanced proliferation to concanavalin A (Con A) and phytohemagglutinin, and changes in disease models thought to be related to thymic abnormalities (13). However, the critical demonstration of differentiation of an immature, nonfunctional T-cell precursor is the development of a cell capable of functional activity. Incubation of cells on monolayers of adherent cells is confounded by the potential problem of selective binding to the adherent cells and the possibility of a selection process that yields the mature subpopulation of thymocytes or spleen cells apart from those affected by soluble factor in this assay.

The ultimate delineation of function of the component parts of thymic stroma requires the dissection of each element by development of cloned pure cell lines. We report here the characterization of a cloned cell line of *thymic origin* as epithelium by morphological, ultrastructural, and biochemical criteria. These cells secrete a factor that promotes at least one functional activity of T cells from immature thymocytes—i.e., production of IL 2.

MATERIALS AND METHODS

Animals. C57BL/6 and (C57BL/6 × DBA/2)F_1 (BDF_1) mice were bred in the animal colony at the University of California at San Diego, La Jolla, and were used as thymus or spleen donors at 4–6 wk of age. AKR/J mice were bred in the animal colony at the Laboratory of Biomedical and Environmental Sciences at the University of California, Los Angeles, and were used as thymus donors for development of the thymus epithelial cell line TEPI.

Factor Production. The TEPI cell line was maintained by weekly passage and growth in Dulbecco's minimal essential medium (DME medium) and 10% fetal calf serum. The growth medium was removed from 5-day cultures and replaced with serum-free DME medium for 24 hr. The 24-hr supernatant served as the source of factor.

IL 2 Assay. Supernatants from thymocytes (6×10^5) stimulated for 24–48 hr with 2 μg of Con A per ml or TEPI su-

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Abbreviations: IL 2, interleukin 2; IL 1, interleukin 1; PNA, peanut agglutinin; TEPI SN, TEPI supernatant; EGF, epidermal growth factor; MLR, mixed lymphocyte reaction; Con A, concanavalin A; TRF, T-cell replacing factor; FITC, fluorescein isothiocyanate; CTL, cytotoxic T lymphocyte.

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pernatant (TEPI SN) (or both) in 0.2 ml of RPMI 1640 and 5% fetal calf serum in 96-well flat-bottom plates were tested for Il 2 activity by using the CTLL-2 T-cell line (14). CTLL-2 cells (5×10^3) were incubated for 24 hr with various dilutions of supernatant. Proliferation was measured by labeling the cells with ^{125}IdUrd for the last 4 hr of culture.

Cytotoxic T Lymphocyte (CTL) Assay. Thymocytes (5×10^6) were cultured in 2 ml of RPMI 1640 supplemented with 5% fetal calf serum, glutamine, penicillin, streptomycin, and 2-mercaptoethanol (50 μM) with 5×10^6 mitomycin-treated spleen cells in CoStar 24-well plates for 5 days at 37°C in a humidified 5% CO_2 in air atmosphere. After 5 days cells were assayed for killing by 4-hr incubation with ^{51}Cr-labeled Con A blast targets. The percent specific isotope release was calculated according to the standard formulation.

T-Cell Replacing Factor (TRF) Activity. The ability of supernatants to replace T cells in the antibody-forming response was determined as described (15). B cells were cultured at 6×10^5 cells per well in the presence of 10–30% IL 2-containing supernatant (FS6.14.13 or AOFS 21.10.9) and 0.01% sheep erythrocytes. After 4 days of culture, direct plaque-forming cells to sheep erythrocytes were determined by the slide modification of the sheep erythrocyte Jerne plaque assay.

Separation of Thymocytes by PNA. Enrichment for PNA^+ thymocytes was performed according to the method described by Kruisbeek and Astaldi (5). An analysis of the PNA^+ population by staining with fluorescein isothiocyanate (FITC)-PNA by using a fluorescence-activated cell sorter (FACS-IV) indicated <4% contamination by PNA^- or low fluorescent cells.

Microscopy. Cell cultures were examined and photographed with a Leitz inverted-phase contrast microscope. For transmission electron microscopy, cells were fixed *in situ* and viewed with a Hitachi HS-8 electron microscope (16).

Chromatography. A molecular weight estimation was done according to the LKB instruction manual. Gel filtration was performed at 4°C with ACA 54 equilibrated with 0.25 M phosphate-buffered saline in a 100 × 2.5 cm column. A 4-ml sample, previously prepared by ammonium sulfate precipitation at 80% saturation of 100 ml of 24-hr TEPI SN and dialyzed extensively against 0.25 M phosphate-buffered saline, was applied to the column. Eight-milliliter fractions were collected. Calibration of the column was done with marker proteins of known molecular weights (bovine serum albumin, ovalbumin, and ribonuclease). Elution positions were determined by UV absorbance at 280 nm.

RESULTS

Isolation of Thymus Epithelium. The TEPI cell line was established by primary culture of 1×10^8 AKR thymocytes in a 60-mm Petri dish in 5 ml of DME medium and 20% fetal calf serum. After 48 hr the thymocytes were gently washed away and the scattered few adherent cells were fed with 50% fresh DME medium containing 20% fetal calf serum and 50% conditioned medium, obtained after centrifugation of the thymocytes. The primary cultures that contained a variety of cell types were maintained by weekly feeding with a similar 50:50 mixture of fresh and conditioned medium. After 4 wk, several isolated colonies of epithelial-like cells covered the plate. At this time a secondary culture was made by transfer of several of these colonies scraped from the primary culture. Growth continued to be slow until the third subculture, when cells began to form a monolayer within 4–5 days. Cloning of the cells by limiting dilution at one cell per well and visual inspection of wells for the presence of individual cells proved to be unsuccessful. Only wells containing more than one cell, usually at least three or four, were observed to grow to confluency.

Epidermal growth factor (EGF) has been described to be a potential inducer of adherent cell mitogenesis (17). We theorized that single epithelial cells are unable to proliferate because of lack of a growth-promoting environment, usually provided by other cells or serum (18). On this basis, we added EGF (kindly provided by Harvey Herschman) at 6 ng/ml to wells containing cells plated in limiting dilution and visually inspected to contain a single cell. Only 34 of 120 wells containing EGF exhibited cell division and eventual confluent cell growth. Initially, five clones exhibiting epithelial-like morphology were grown out and the supernatants tested for ability to enhance alloreactivity in whole thymocyte populations.

Morphology of TEPI Cell Line. Morphologically, these cells display characteristics consistent with epithelium. They have a large (50 μm) polygonal shape with a centrally located round nucleus and in monolayer culture give a "cobblestone appearance" (Fig. 1). The nucleus has dispersed chromatin and several nucleoli. The cytoplasm has a network of rough endoplasmic reticulum, well-developed Golgi apparatus, and many vacuoles, typical of a secretory cell. In addition, the cytoplasm contains many tonofibrils and the plasma membranes of adjacent cells display specialized associations—i.e., desmosomes or tight junctions (Fig. 2). Mature type C viruses were seen budding from many of the TEPI cells. To further analyze the cells for epithelial characteristics, the cells were stained with antibody to fibronectin, an extracellular protein component of basement membrane thought to be of importance in cellular morphogenesis (19). As shown in Fig. 3, the TEPI dislayed an extracellular matrix of fibronectin similar to that of other stromal cells.

An additional characteristic attributed to reticuloepithelial cells of the thymus has been the expression of Ia antigen. Indirect immunofluorescent staining of the cells by using a monoclonal anti-Ia^k antibody revealed Ia antigen on their surface (data not shown). Furthermore, these cells were capable of stimulating a long-term T-cell line specifically recognizing Ia^k to release TCRF (Table 1).

Functional Characteristics of TEPI *in Vitro*. Supernatants from confluent TEPI monolayers were tested for their capacity to promote thymocyte functional activity. In the first set of experiments we tested the ability of TEPI SN to augment the CTL response of thymocytes to allogeneic major histocompatibility complex antigen.

One prominent intrathymic differentiative event is the transition from a non-MLR reactive cell to a highly MLR-competent T cell. Presumably this reflects the maturation of helper

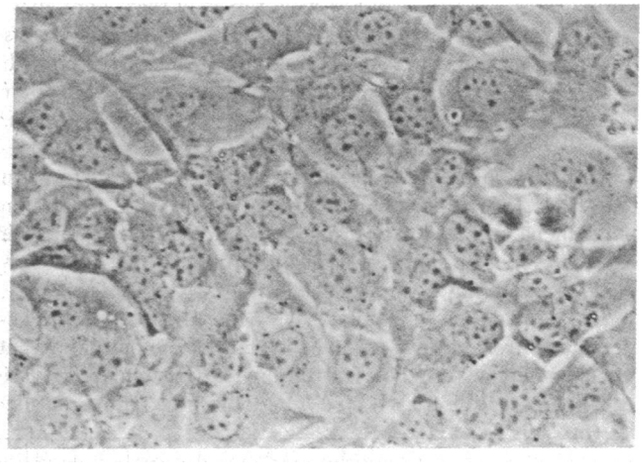

FIG. 1. Low-power photomicrograph of a 5-day confluent monolayer culture of TEPI. (×300.)

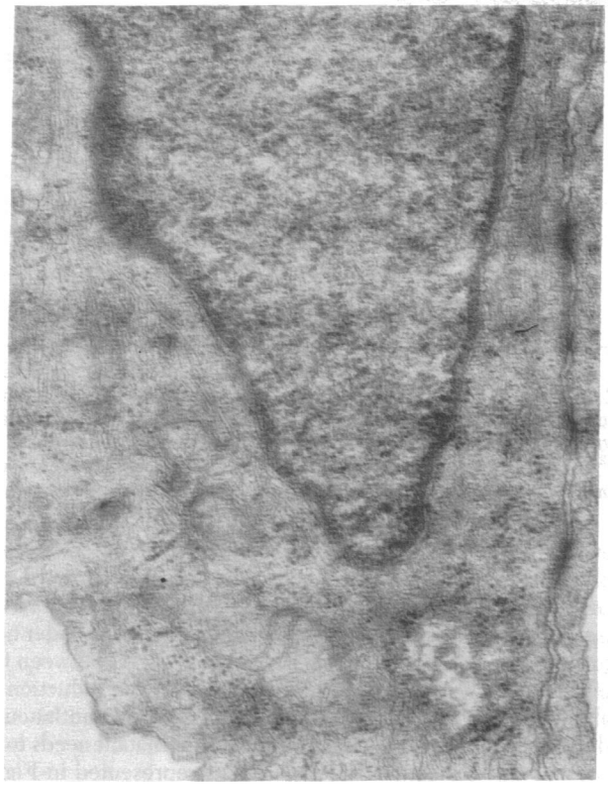

FIG. 2. Electron micrograph of a 5-day confluent monolayer culture of TEPI. (×9,600.)

T-cell function, which then can provide the already present CTL precursors with the requisite signal to develop into killer T cells. The results, illustrated in Fig. 4, show an increase in the amount of CTL activity after 5 days of culture in the presence of TEPI SN. This activity is also provided by supernatants from a known IL 2-producing hybridoma. Thus, TEPI SN activity is comparable to the known ability of IL 2 to substitute for help in CTL induction. However, it was demonstrated that TEPI SN has no activity in the T-cell growth factor assay (Table 2).

To further distinguish TEPI SN from IL 2, another experimental protocol was employed. PNA^+ thymocytes were preincubated for 24 hr with IL 2 or TEPI, washed three times, and

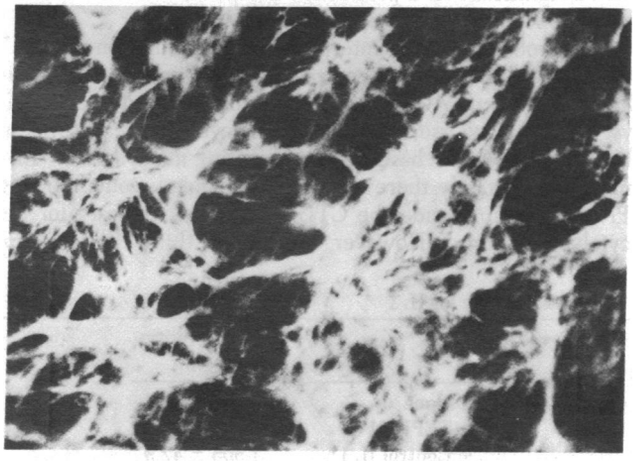

FIG. 3. Indirect immunofluorescent staining for fibronectin by using a rabbit anti-mouse fibronectin and a fluorescein-conjugated goat anti-rabbit immunoglobulin of a TEPI monolayer. (The identical microscope field as in Fig. 1 is shown; ×300.)

Table 1. Demonstration of Ia^k expression by TEPI by ability to stimulate C.C3.11.75 to the production of helper factor (DL)TRF

Addition to culture for the generation of (DL)TRF	Plaque-forming cells per culture*
No factor	<10
C.C3.11.75 SN†	
Stimulated with BALB.K spleen	200
Stimulated with TEPI	270
Stimulated with L929	<10

*The (DL)TRF was assayed in cultures containing 6×10^5 spleen cells treated with anti-Thy 1.2 and complement and stimulated *in vitro* with sheep erythrocytes.

†C.C3.11.75 is a long-term CTL line specific for Ia^k and releases TRF upon stimulation (15).

then cultured in mixed lymphocyte culture (Fig. 5). It can be seen that preincubation with TEPI SN increased the subsequent CTL response, whereas preincubation with IL 2 was almost without effect. This result suggested that the CTL-enhancing activity was substituting for the exogenous IL 2 requirement, possibly by inducing IL 2 production.

Therefore, we tested the activity of TEPI SN on various cell populations in an assay in which the Con A induction of IL 2 production was measured. Data in Table 3 indicate a significant increase in IL 2 production by PNA^+ thymocytes in the presence of TEPI SN. In contrast, the effect of TEPI SN on other cell populations is slightly negative or not significant.

Preliminary Biochemical Characterization of TEPI SN. To exclude the possibility that the CTL-enhancing activity of TEPI SN was not due to interleukin 1 (IL 1), which is known to promote IL 2 production (20), it was tested in the costimulator assay (21) and shown to be negative (Table 4). A further distinction of TEPI SN factor from IL 1 was made by gel chro-

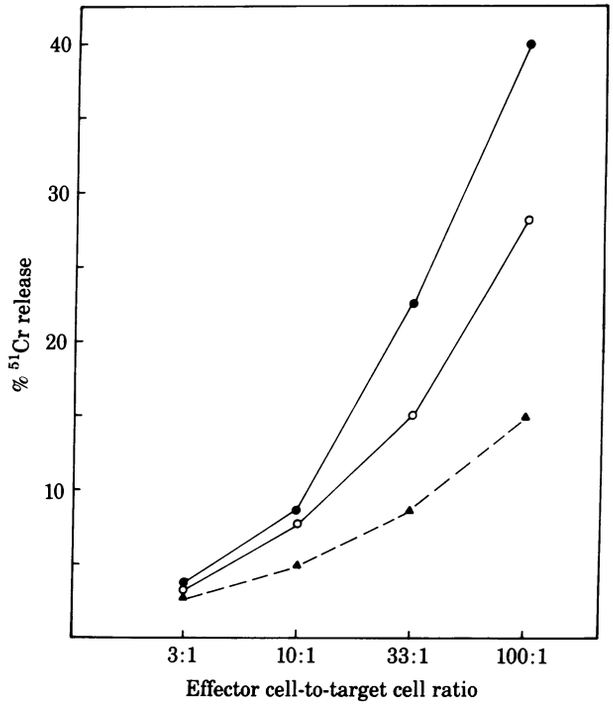

FIG. 4. Assay for augmentation of thymocyte allo-CTL. The responses of PNA^+ thymocytes in the presence of FS6.14.13 SN (IL 2) (10%, vol/vol) or crude TEPI SN (10%, vol/vol) are indicated by ○ or ●, respectively. The response of cells without additions to the culture is indicated by ▲.

Table 2. Lack of IL 2 activity in TEPI SN

Source of SN	Radioactivity, cpm
TEPI	272 ± 61.3
MAC-2	799 ± 111
Mls-BW	3,818 ± 762
Medium alone	164 ± 44.4

CTLL-2 cells (20) (5×10^3) were incubated for 48 hr with 2.5% (vol/vol) supernatant from TEPI, MAC-2 (a thymic macrophage line; unpublished data), or Mls-BW (a T-cell hybridoma; unpublished data). Cultures were labeled with ^{125}IdUrd for the last 4 hr. Results are expressed as mean ± SD.

matography. Fig. 6 represents a profile of the IL 2-inducing activity of TEPI SN. The major peak of activity is in the M_r 50,000 range.

DISCUSSION

A cloned cell line of nonlymphoid cells from the thymus has been characterized as epithelial in origin. The TEPI line displays the polygonal morphology with a centrally located nucleus with many nucleoli described by others (6). Histochemically, the TEPI cell line exhibits other characteristics that would exclude other cell types of similar morphology. In particular, the presence of an extracellular fibronectin matrix is a feature that distinguishes TEPI from macrophages. Ultrastructurally, the ability of TEPI to form membrane associations between adjacent cells would argue against it being a fibroblast line. Furthermore, the expression of Ia antigen has not been reported for a fibroblast line, whereas, Ia expression is an antigenic marker for thymic epithelial cells (22). Based upon these physical data we conclude that TEPI is in fact epithelial in origin.

It is clear that the culture supernatant from TEPI is capable of inducing strong alloreactivity in PNA$^+$ thymocytes. However, it is well known that thymus contains CTL precursors but is lacking in functional helper cells. The lack of help can be circumvented by the addition of IL 2 (12). The first question raised is whether TEPI SN is acting in a manner similar or identical to IL 2. Previously, others (12) have distinguished between the effects of epithelial supernatant and IL 2 in the induction of allo-CTL from thymocytes. IL 2 must be present continuously during the reaction, whereas epithelial supernatant needs to be present only for 24 hr. The experiment represented in Fig. 5 shows that preincubation with TEPI SN can influence the subsequent reactivity of thymocytes to alloantigen, whereas IL 2 alone has only a slight effect. The small response of PNA$^+$ thymocytes alone or the small effect of IL 2 may be due to the stimulator population providing a small amount of IL 2. Another problem is the question of selective survival of mature cells during the 24-hr incubation. However, one would expect that if mature PNA$^-$ cells were surviving, IL 2 would have the greatest effect, because it is known to be a T-cell growth factor. Although we attempted to obviate this by using F_1 stimulators, it is known that BDF_1 responds well to parental C57BL/6. This suggested that TEPI SN is effecting the maturation of a helper cell, which can subsequently deliver the proper signal to the CTL precursors in the presence of antigen. In fact, this notion is supported by the results presented in Table 3. Addition of TEPI SN to PNA$^+$ thymocytes in the presence of Con A stimulated significant IL 2 production compared to PNA$^+$ thymocytes alone.

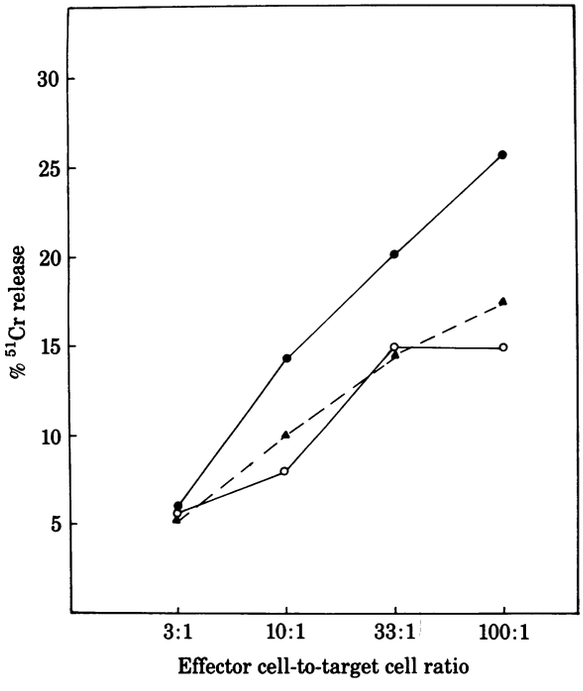

FIG. 5. Comparison of the effect of IL 2 and TEPI SN. CTL responses of PNA$^+$ thymocytes to alloantigen when preincubated for 24 hr in the presence of TEPI SN are indicated by ●. Responses of cells preincubated for 24 hr in the presence of FS6.14.13 (IL 2) are indicated by ○. Control responses are represented by ▲.

Table 3. Effect of TEPI SN on IL 2 production by various cell populations

Cells	Radioactivity, cpm
Spleen cells	3,750 ± 319
Spleen cells + TEPI SN	3,390 ± 121
Thymocytes	2,316 ± 149
Thymocytes + TEPI SN	2,217 ± 122
PNA$^+$ thymocytes	527 ± 34
PNA$^+$ thymocytes + TEPI SN	1,072 ± 21
PNA$^-$ thymocytes	3,921 ± 291
PNA$^-$ thymocytes + TEPI SN	3,548 ± 170

Lymphoid cells (6×10^6) were cultured in microwells for 48 hr with 2 μg of Con A per ml in the presence or absence of TEPI SN. The culture supernatants of these cultures were assayed for T-cell growth factor (IL 2) by using CTL line CTLL-2 (20). The results are expressed as ^{125}IdUrd incorporation (cpm) per 5×10^3 CTLL-2 cells. These data, expressed as mean ± SD, are representative of several experiments.

One problem with the interpretation of maturation of PNA$^+$ thymocytes has recently been raised by the use of limiting-dilution analysis (LDA) (23). According to limiting-dilution analysis of CTL precursors in PNA$^+$ thymocytes, all reactivity can be explained on the basis of contaminating mature PNA$^-$ thymocytes. Certainly there are probably enough contaminating PNA$^-$ cells to provide the CTL precursors necessary for the cytolytic response. However, we do not think the apparent

Table 4. Assay for IL 1

	Radioactivity, cpm
Thy alone	149 ± 23.6
+ TEPI SN	260 ± 39.9
+ Control IL 1*	1,905 ± 42.9

Thymocytes (5×10^4) were cultured for 48 hr with 2 μg of Con A per ml. ^{125}I incorporation was determined as cpm by a pulse label during the last 4 hr of culture.

*Supernatant from an IL 1-producing cell line $P388D_1$.

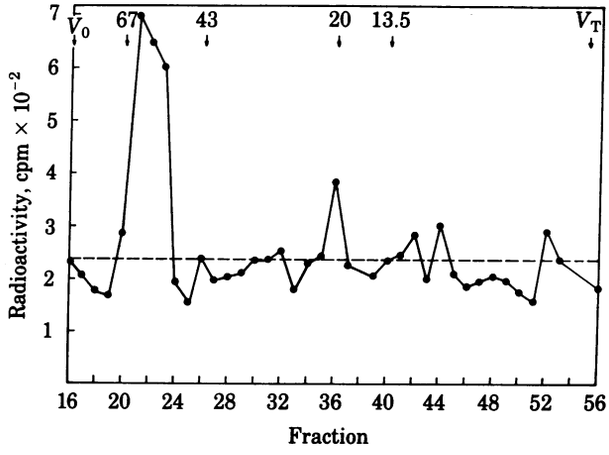

FIG. 6. Profile of TEPI SN IL 2-inducing activity separated on ACA 54 gel. PNA^+ thymocytes (6×10^5) were cultured in microwells with 2 μg of Con A per ml in the presence of 10% (vol/vol) final concentration of column fractions for 48 hr. Supernatants from these cultures were then tested for IL 2 activity by stimulation of CTLL-2 proliferation. The dashed line represents the IL 2 activity of supernatant of PNA^+ thymocytes stimulated with Con A alone. Molecular weights are shown as $M_r \times 10^{-3}$.

stimulation of help with TEPI SN is explicable on the basis of effects upon residual PNA^- thymocytes. First, the frequency of PNA^- IL 2 producer in PNA^+ thymocytes in the presence of TEPI SN is lower than that of 1.2×10^4 PNA^- thymocytes, present in 3×10^5 PNA^+ cells based upon a maximal 4% contamination determined by fluorescence-activated cell sorter analysis (data not shown). Second, data in Table 2 indicate that TEPI SN has no significant effect on PNA^- thymocytes. Third, these data would also explain why the IL 2 production by whole thymocytes in the presence of TEPI SN is not an arithmetic sum of IL 2 produced by PNA^- and PNA^+ thymocytes (Table 3). There are negative effects occurring in mixed populations of cells. Whether this is due to suppression or the simultaneous stimulation of IL 2 users cannot be distinguished at this time.

The possibility that this activity was similar to the known ability of IL 1 to induce IL 2 release was tested in two ways. Initially, TEPI SN was tested for activity in the costimulator assay and found to be negative. Second, and more conclusively, the activity in TEPI SN that chromatographs in the M_r 50,000 range was in contrast to the M_r 15,000 reported for IL 1 (24) (Fig. 6).

How do these observations fit into the scheme of T-cell maturation?

Several factors of thymic origin have been described (25), which have been shown to induce membrane changes or antigenic changes in lymphoid populations. However, none has consistently demonstrated the functional development of a T-cell subset. It is likely that the apparent confusion in describing thymic factors reflects the complexity and multiplicity of thymic functions. The process of T-cell differentiation and determination of the self-recognition phenotype probably requires several discrete steps leading to the development of different functional subsets of T cells (26). Each point along the pathway of maturation and selection may require interaction with the soluble product of a particular cell type within the thymic stroma. Thus, no single factor by itself will be capable of total reconstitution of thymic function. Additional support for this concept has been given by recent data indicating that highly purified IL 2, by itself, is incapable of augmenting PNA^+ alloresponsiveness to heat-inactivated cells (27). The interpretation rendered by the authors from such data is that another factor probably derived from the stimulator spleen population is required.

Within this framework of thymic function we have described a cloned cell line of thymic origin that produces a factor capable of inducing responsiveness to the induction of IL 2 production within a cell population, PNA^+ thymocytes, unable to do so prior to exposure to TEPI SN. Implicit in the result is the maturation of one subset of immature thymocytes to a functional T cell. However, we cannot exclude the possibility that TEPI SN is not acting indirectly by inducing production of another factor by a third cell type. Further proof of such maturation will require analysis for expression of differentiation antigens. The availability of the TEPI cell line that produces a factor capable of inducing IL 2 production by immature thymocytes will allow further studies in the understanding of thymus function.

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