Identification of a T Helper Cell–Derived Lymphokine That Activates Resting T Lymphocytes

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A novel lymphokine with apparent molecular size of 10 to 12 kilodaltons is secreted from helper T cell clones within hours after cross-linking their T cell antigen–MHC (major histocompatibility complex) receptors (T3-Ti). This lymphokine, termed interleukin-4A (IL-4A), stimulates resting lymphocytes by binding to a surface component (or components) of the alternative T11 pathway and subsequently by inducing interleukin-2 (IL-2) receptors. The activation process is neither dependent on antigen specificities of the recruited population or the presence of macrophages. It appears, therefore, that IL-4A is a mediator involved in amplifying the T cell immune response.

ACTIVATION OF RESTING HUMAN T LYMPHOCYTES IS INITIated through either the antigen-MHC (major histocompatibility complex) receptor (T3-Ti) or the T11 structure (*1*). The T3-Ti structure is a molecular complex comprised of five subunits: a 90-kilodalton clonotypic Ti alpha-beta heterodimer and three noncovalently associated 20- to 25-kD T3 molecules (gamma, delta, and epsilon) (*2*). Variability among individual T cell receptor Ti α and β subunits, as with immunoglobulin (Ig) genes, results from germline diversification of the variable (V), diversity (D), and joining (J) elements that encode them and combinatorial events linking these discontinuous gene segments during ontogeny (*3*). This, in principle, creates a large array of ligand (antigen-MHC) binding sites. Although the invariant nature of the T3 subunits precludes their involvement in antigen recognition, the rather lengthy cytoplasmic domains of the 20-kD subunits relative to the short Ti α and β intracytoplasmic domains suggests that the T3 molecules are involved in signal transduction (*4*). Unlike the T3-Ti complex, the T11 surface molecule, is a single chain 50-kD structure, has no known polymorphic determinants and, as such, does not appear to impart antigen specificity (*1, 5*). Moreover, T11 is distinct from the T3-Ti structure in that it is more preserved phylogenetically (*6*) and appears earlier during intrathymic ontogeny (*1, 7*). Possibly, T11 represents a primitive T cell surface activation structure that subsequently evolved into a more specialized multichain T3-Ti molecular complex; a relation not dissimilar to that between insulin-like growth factor receptors and insulin receptor.

Whether or not the T11 molecule is the primitive T lineage receptor, its function is not vestigial: stimulation of T cells by T11, as by the T cell antigen–MHC receptor results in activation of the T cell program whether effector or regulatory in nature (*1*). Thus, the pathway involving T11 has been termed the alternative pathway of activation. Early events, triggered by either pathway include transmembrane calcium flux, sodium-proton exchange activation, and alterations of phosphatidylinositol (*8*). Through intermediate steps yet to be elucidated, interleukin-2 (IL-2) gene and IL-2 receptor gene activation follow in both pathways (*1, 9*). Once IL-2 is secreted and binds to a critical number of IL-2 receptors, DNA synthesis and cell mitosis occur. Furthermore, in the case of the T11 pathway, activation can lead to induction of IL-2 receptor expression on stage I and stage II thymocytes (*10*).

If the T11 pathway is not a relic within the mature T cell compartment, it must have some function. One possibility follows from the immunobiology of T cell proliferation; namely, antigenic stimulation of a T lymphocyte population induces both antigen-specific T cell response and proliferation of T cells with specificities unrelated to the stimulating antigen (*11*). For such a phenomenon to occur a priori, there must be a mechanism by which antigen-MHC interaction with T3-Ti on one antigen-specific T cell is able to activate other resting T lymphocytes regardless of antigen-MHC specificity. We propose that the T11 pathway may serve in such recruitment of resting T cells. Given that the T11 pathway, unlike the T3-Ti pathway, has no requirement for macrophages and IL-1 (*1, 12*), fewer constraints would be placed on the activation process. The above hypothesis is predicated on the supposition that a natural ligand of the surface T11 pathway is released from T3-Ti triggered T cells and that this presumptive lymphokine in turn activates resting T lymphocytes.

To examine this possibility, we tested the capacity of one representative inducer T cell clone, termed 5B, to produce such an activating molecule or molecules after T3-Ti stimulation. Cloned cells (1×10^6 per milliliter) were placed in polypropylene tubes and stimulated for varying periods of time (8 to 48 hours) with Sepharose-linked monoclonal antibodies (*1*). Culture supernatants derived from these stimulated 5B cells were then harvested and passed through 0.45-μm filters. The supernatant was added to a rigorously purified T cell population and the mixture was then incubated for 1 to 5 days. Subsequent proliferation was assessed by incorporation of tritiated thymidine (TdR) (*13, 14*).

Supernatants generated from 5B cells that had been cultured for

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as little as 8 hours with the antibody to the receptor (anti-clonotypic) (anti-Ti_3) linked to Sepharose-induced proliferation of resting T cells (Fig. 1A). This activity was maximal in the 5B supernatant after 24 hours of stimulation with anti-Ti_3 bound to Sepharose. After the cells were stimulated for 48 hours, the proliferation was definite but suboptimal. The Sepharose-bound antibodies to the 62-kD T4 T cell surface protein structure expressed on 5B did not result in [^{3}H]TdR incorporation. In the absence of anti-Ti stimulation, no constitutive activity was noted (Fig. 1). Activity was observed when the stimulated T cell clones and resting T lymphocytes were derived from autologous (Fig. 1) or allogeneic pairs (*15*). The effect of the anti-Ti–stimulated supernatant on resting T cells was maximal after 4 days of culture.

The effect of the anti-Ti Sepharose–triggered 5B supernatant was not simply a result of IL-2 secretion; in the presence of exogenous IL-2 (>5 to 50 U/ml), whether recombinant or natural material (*16*), no proliferation of resting T cells was observed, a result consistent with earlier studies indicating that resting T cells lack IL-2 receptors and that IL-2 does not itself induce IL-2 receptor expression on the cells (*9*). Treatment with lytic complement in the presence of a mixture of antibodies to Ia antigen, transferrin receptor, Mo1, and IL-2 receptor (legend to Fig. 1) removes any residual in vivo activated T cells contained within the Ficoll-Hypaque preparation from peripheral blood lymphocytes.

To determine whether the above activity occurred only in the 5B clone, we tested the supernatants from other T cells. Four T4+ helper clones, one T8+ cytotoxic clone, one T4+ cytotoxic clone, and one T8+ suppressor clone were examined (Fig. 1B). In each case, monoclonal anticlonotypic antibodies specific for the Ti molecule of individual clonal populations have been described (*17*). As with 5B, clones were stimulated in vitro with antibodies to specific clonotypes (anti-Ti) bound to Sepharose for 24 hours and added to a resting T cell population. As a control, supernatants were examined from individual clones that had been cultured with anti-Ti (of the inappropriate specificity) bound to Sepharose (unstimulated) and in some cases, supplemented with 10 units of nonrecombinant IL-2 (IL-2). In each case, [^{3}H]TdR incorporation was tested after 4 days.

Relevant anti-Ti–stimulated cell supernatants from all four helper clones (5B, AC3, RW17C, AA18) caused proliferation of resting T cells (Fig. 1B). In contrast, no such activity was obtained from either T4+ or T8+ cytotoxic clones (AA8, AA14) or the T8+ suppressor clone 20a6. As with the 5B clone, supernatants from unstimulated cells (±IL-2) did not induce proliferation of resting T lymphocytes. Clones 5B and AA8 were stimulated with the identical clonotypic monoclonal antibody, anti-Ti_3, since they share the same V beta REX gene segment product to which the antibody is directed (*18*); nevertheless, the ability to induce resting T cell activation is restricted to the 5B supernatant. From these data, it would appear that the observed activity is a function of the T4+ helper population and not cytotoxic or suppressor populations.

The ability of helper T cell supernatants (T_{HSN}) to induce activation of resting T cells is reminiscent of a combination of monoclonal antibodies to two defined epitopes termed $T11_2$ and $T11_3$ to stimulate T cell proliferation. To determine whether anti-T11 antibodies and T_{HSN} mediate their effects through a common pathway, we sought additional analogies. We found that T_{HSN} could induce resting T cells to proliferate, as in the case of antibodies to T11, in the virtual absence of macrophages (Fig. 2A). Thus, whether or not macrophages are present in the rigorously purified T cell population has no effect on the subsequent response of resting T cells to any concentration of supernatant tested. In contrast, removal of macrophages from this same preparation of T lymphocytes abrogated proliferation to the macrophage-dependent IgG2 murine monoclonal anti-T3, RW2-8C8 (*19*).

Given that surface T11 stimulation with anti-T11 results in IL-2 receptor induction on thymocytes and T cells, we next determined whether T_{HSN} would induce IL-2 receptor expression on these cells as well. The T cell clone 5B was stimulated with anti-Ti_3 under conditions described above, supernatants were harvested and added to resting T cells, and the IL-2 receptor expression of the stimulated T cells was examined with a direct immunofluorescence assay (Epics V cell sorter). Culture of resting T cells for 4 days with anti-Ti_3 Sepharose stimulated 5B supernatants (day 4 plus SN) resulted in detectable IL-2 receptor expression on more than 35 percent of resting T lymphocytes. In contrast, fresh uncultured T cells or T cells cultured for 4 days with an anti-T4 Sepharose induced 5B supernatant (day 4), expressed no detectable IL-2 receptors (<1 percent).

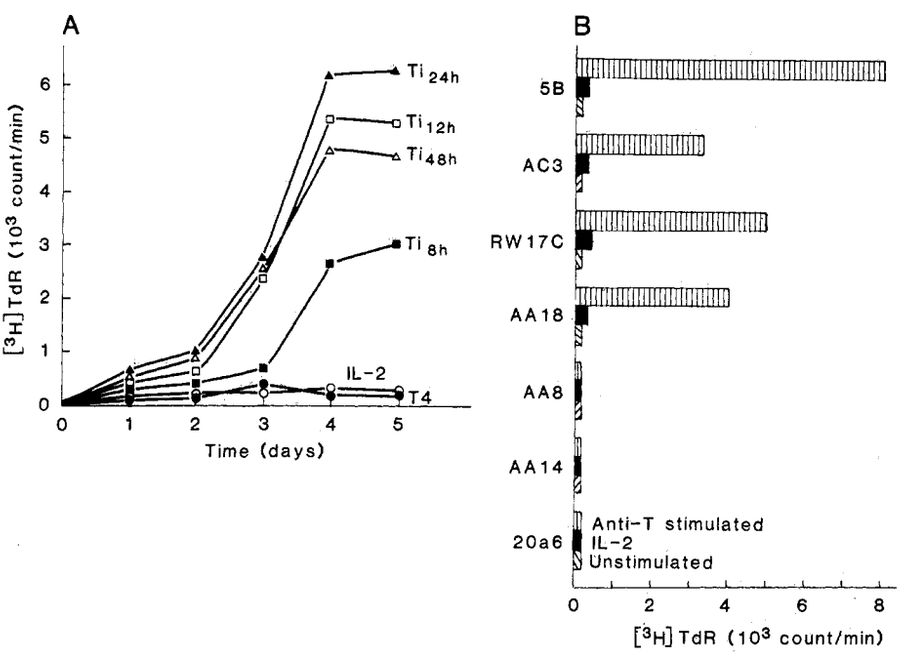

Fig. 1. Cross-linking of T cell antigen-MHC receptors on helper T cells induces activity in supernatants that stimulates resting T lymphocytes. (A) Kinetics of proliferation of resting human T cells to purified IL-2 (10 U/ml) or supernatants derived from the 5B helper T cell clone after stimulation with anti-Ti (anti-Ti_3) for 8, 12, 24, or 48 hours (Ti_{8h} to Ti_{48h}) or anti-T4 Sepharose (24-hour supernatant only shown, T4). Ordinate represents [^{3}H]TdR and abscissa, the day on which T cells were labeled with [^{3}H]TdR. Results shown represent the mean of triplicate samples. Standard deviations were <10 percent in all experimental triplicates. (B) Capacity of specific anti-Ti stimulated (▤), IL-2 supplemented (■), or unstimulated (▨) supernatants from individual T cell clones to induce resting T cell proliferation. The production and characterization of T cell clones and monoclonal antibodies have been described (*17, 18*). 5B, AC3, RW17C, and AA18 are T3+ T4+ T11+ helper clones, as assessed by their ability to induce B cells to secrete Ig in the presence of an appropriate stimulus. AA8 (T3+ T4+ T11+) and AA14 (T3+ T8+ T11+) are antigen MHC restricted cytotoxic clones, and 20a6 is a T3+ T8+ T11+ noncytotoxic clone able to suppress Ig secretion from activated B cells.

Table 1. Induction of human thymocyte IL-2 responsiveness by T_{HSN}. Human thymocytes were obtained from the thymuses of 2- to 4-month-old newborns (*10*). The response to purified IL-2 at 10 U/ml (*16*), T_{HSN} (25 percent, by volume), or combinations of IL-2 and T_{HSN} or IL-2 plus IL-1 are shown as mean ± SD of triplicate samples. Purified human IL-1 was purchased from Cistron (Pine Brook, NJ) (10 U/ml). In proliferative assays we used 50,000 thymocytes with culture conditions as described for T cells. Samples were labeled with [^{3}H]TdR on day 4 and harvested 16 hours later. IL-1 activity at this concentration was optimal in the standard submitogenic phytohemagglutinin murine thymocyte assay. In experiments where rIL-1 (*27*) at 30 ng/ml alone or in conjunction with IL-2 were tested, no thymocyte proliferation was observed.

Stimulus	Unfractionated thymocytes (count/min)	
	Exp. 1	Exp. 2
Media	225 ± 32	436 ± 83
IL-2	396 ± 45	699 ± 34
T_{HSN}	2,253 ± 211	1,385 ± 27
IL-2 + T_{HSN}	12,677 ± 830	18,555 ± 948
IL-2 + IL-1	367 ± 18	933 ± 62

Similar results were also obtained with T_{HSN} on thymocyte populations (*20*).

In addition to the phenotype evidence for expression of surface IL-2 receptors on T_{HSN}-incubated resting T cells, functional data also provided support for the physiologic integrity of these IL-2 receptors. The addition of increasing concentrations of recombinant IL-2 to the T_{HSN} plus resting T cell co-culture results in a dose-dependent increase in the proliferation (Fig. 2B). This response can be inhibited with monoclonal antibodies to either IL-2 or the IL-2 receptor. Furthermore, T_{HSN} is also capable of inducing IL-2 responsiveness among human thymocytes (Table 1). In each of two representative experiments, little if any proliferation resulting from IL-2 alone was observed among unfractionated thymocytes (10 U/ml, purified nonrecombinant gives less than 700 count/min compared to 225 to 436 count/min in the media control). In contrast, T_{HSN} from the 5B T cell clone in conjunction with 10 U/ml of IL-2 resulted in substantial thymocyte proliferation. As shown, even IL-1 at optimal concentrations for the IL-1 thymocyte proliferation assay (semipurified, 10 U/ml) could not replace the requirement for T_{HSN} in induction of IL-2 responsiveness among thymocytes (Table 1). The capacity of T_{HSN} to activate resting T cells and thymocytes to express IL-2 receptors in the absence of IL-1 is certainly also consistent with known IL-1 independence of the T11 pathway.

To directly link the T_{HSN} activity to the surface T11 structure, we performed a series of absorption studies with T11+ and T11− lymphoid cells. We reasoned that absorption with a T11+ lymphoid cell population would remove the T_{HSN} lymphokine and hence deplete functional activity in the resting T cell assay. On the contrary, absorption with T11− cells should have no effect. For this purpose, we examined the differential capacity of cells from the T11+ human T cell tumor line (REX) and T11-REX variants created by selective mutagenesis (legend to Fig. 2C) to remove T_{HSN} activity. These variants were produced and used to help eliminate the possibility that undefined variables might account for differential absorption.

T_{HSN} induces proliferation of resting T cells before, but not after, absorption with the T11+ REX tumor population (8800 compared

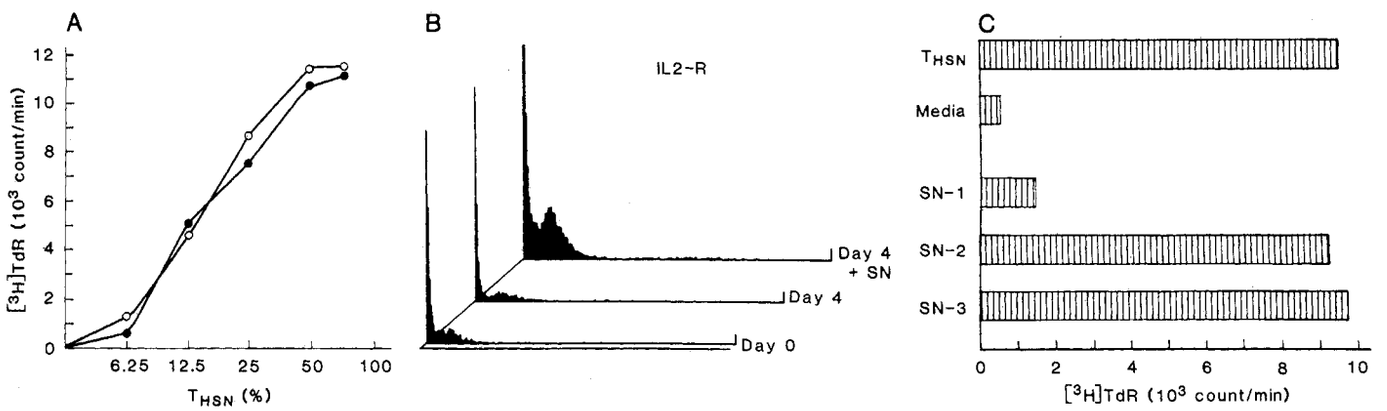

Fig. 2. Relation of T_{HSN} lymphokine to the T11 pathway of T cell activation. (A) Macrophage independence of T_{HSN} activity. The proliferative response of resting T cells to increasing concentrations (by volume) of T_{HSN} was tested in the presence (●) and absence (○) of 5 percent macrophages prepared as described (*1*). (B) T_{HSN} induces IL-2 receptor expression on resting T lymphocytes: 40,000 purified T lymphocytes were examined (Epics V cell sorter; Coulter Electronics, Hialeah, FL) for IL-2 receptor expression by means of direct immunofluorescence with fluorescein isothiocyanate (FITC)-labeled anti-IL-2R1, a monoclonal antibody, at a 1:50 dilution at time 0 (day 0) or 4 days of culture in the presence (day 4 + SN) or absence (day 4) of T_{HSN}. As shown, IL-2 receptor expression appeared on more than 35 percent of cells only after culture with T_{HSN}. Preparation of direct immunofluorescence samples was as described (*10*). Representative responses of 50,000 resting T cells to recombinant IL-2 (rIL-2) (Biogen, Cambridge, MA): 0.5 U/ml, 300 count/min; 5 U/ml, 330 count/min; 50 U/ml, 310 count/min. Representative response of T cells to the combination of various concentrations of IL-2 plus T_{HSN} 33 percent by volume was: IL-2 (0.5 U/ml + T_{HSN}) 9250 count/min; IL-2 (5 U/ml + T_{HSN}) 24,600 count/min; and IL-2 (50 U/ml + T_{HSN}) 42,100 count/min. Anti-IL-2 monoclonal antibody (DMS1) at 100 μg/ml as well as anti-IL-2R1 at 100 μg/ml inhibited the response mediated by IL-2 (5 U/ml) + T_{HSN} by >75 percent. Isotype controls (IgG1 and IgG2A) had no effect. (C) Absorption of T_{HSN} activity by the T11+ REX tumor population but not its T11− clonal mutants. T_{HSN} from 1 ml of 5B cells stimulated by anti-Ti were absorbed with 50 × 10^6 T11+ REX cells (SN-1) or either of two T11− REX mutants (SN-2 and SN-3) at 4°C for 1 hour in a rotating apparatus (Labindustries, Berkeley, CA). Subsequently, culture supernatants were tested for their ability to induce proliferation of resting T cells and compared with unadsorbed T_{HSN} or supernatant derived from the unstimulated 5B clone (media). Methods are as described in the legend to Fig. 1 and in (*13*, *14*). For mutagenesis, 1 × 10^7 cells from the human T cell tumor line REX were irradiated with 300 rads in a gamma cell 1000 irradiator (Atomic Energy of Canada Ltd., Canada) with cesium-137 as a source. The T11− fraction was then enriched by complement-mediated lysis with anti-$T11_1$ (7T4-7E10) and anti-$T11_2$ (101D2-4C1) at a 1:100 dilution. The lysis procedure was repeated four times. The enriched T11− fraction was then sorted out on an Epics V cell sorter and the sorted T11− cells were cloned by limiting dilution method at a concentration of 0.3 cells per well in V bottom 96-well plates (Linbro, Flow Labs, McLean, VA) in the presence of irradiated feeder layers (1 × 10^5 cells per well) in RPMI 1640 medium containing 5 percent horse serum (JR Scientific, Woodland, CA) supplemented with 200 m*M* L-glutamine and 1 percent penicillin-streptomycin. The growing clones were tested for the lack of $T11_1$ and $T11_2$ molecule surface expression by means of indirect immunofluorescence. Of 23 clones that were tested, 21 were negative for surface expression of the T11 molecule.

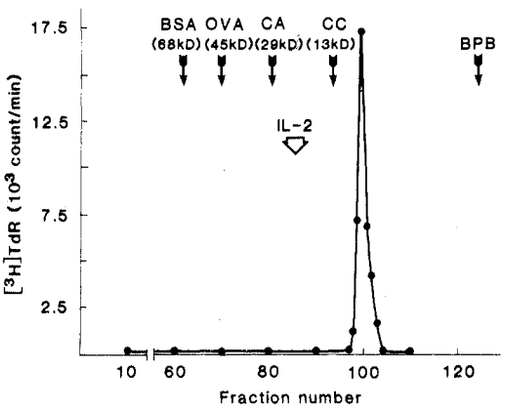

Fig. 3. Sephadex G-100 size fractionation of clone 5B supernatants generated in serum-free media. 5B clone cells (50×10^6) were stimulated in serum-free media containing 1 percent bovine serum albumin (BSA) (Sigma, St. Louis, MO). Subsequently, the supernatant was subjected to precipitation with a 90 percent saturation of ammonium sulfate for 2 hours at 4°C. The precipitate was harvested by centrifugation at 10,000*g* for 30 minutes (Sorvall SS.34 rotor, 4°C), dissolved to a final volume of 5 ml in cold phosphate-buffered saline containing PEG-8000 (0.1 mg/ml), further centrifuged, and then loaded onto a column of Sephadex G-100 (2.6 by 95 cm) and eluted in the cold with the same buffer. Fractions of eluate (4 ml) were collected which were subsequently dialyzed at 4°C against RPMI 1640 medium in Spectrapor dialysis tubing (cutoff 6000 to 8000 daltons) (Spectrum Medical Industries, Los Angeles, CA). After dialysis each fraction was assayed for IL-4A activity as described above in the absence and presence of added recombinant IL-2 (5 U/ml). T_{HSN} activity of individual Sephadex G-100 fractions was measured as 10^{-3} count/min [^{3}H]TdR incorporated into resting peripheral T cells in the presence of 5 U/ml recombinant IL-2 (Biogen). A sharp peak of activity in the fraction corresponding to a molecular size of approximately 10,000 to 12,000 daltons is evident. Column calibration was determined with BSA, ovalbumin (OVA), carbonic anhydrase (CA), cytochrome c (CC), and bromphenol blue (BPB). The open arrowhead indicates the position of IL-2 activity as tested in standard IL-2 assay with the AC3 clone. Elution positions were precisely measured with a Uvicord II (LKB, Gaithersburg, MD).

to 1480 count/min) (see SN-1 in Fig. 2C). In contrast, no activity is removed from the 5B supernatants triggered by anti-Ti when absorbed with either of two representative T11− clonal REX mutants (SN-2 and SN-3). Since these tumor variants (T1+ T3+ T4+ T6+ T8+ T11− T12+ IL2R−) are clonal derivatives of the REX parent (T1+ T3+ T4+ T6+ T8+ T11+ T12+ IL2R−) and differ only by expression of T11, the results provide support for the possibility that the active T_{HSN} fraction binds to the T11 structure. Selection for T3− REX mutants with anti-T3 and complement (leading to loss of the T3-Ti complex but maintenance of the T11 structure) resulted in cells that were indistinguishable from the T11+ REX parent with regard to their effects on T_{HSN}. This latter result indicates that T_{HSN} activity does not bind to T cells via the T3-Ti nominal antigen-MHC receptor complex. Furthermore, absorption with other T11+ T lineage populations (unfractionated peripheral T cells, T4+ T cells, T8+ T cells, or CEM cells) removed T_{HSN} activity, whereas absorption with T11− populations (Molt-4, HSB-2, and the B lymphoblastoid line Laz 509) did not. Thus removal of T_{HSN} activity correlated precisely with T11 expression of the population utilized. From this analysis, we cannot rule out the possibility that a structure physically associated with T11 but not T11 itself might be adsorbing the T_{HSN} activity.

In order to obtain some molecular information about the T11-associated activity in T_{HSN}, we stimulated the 5B helper T cell clone in serum-free media containing 1 percent bovine serum albumin (BSA). Subsequently, serum-free culture supernatant was subjected to a 90 percent saturation with ammonium sulfate, and the resulting precipitate was fractionated on Sephadex G-100 (legend to Fig. 3). Fractions of eluate (4 ml) were collected, dialyzed at 4°C against RPMI 1640 medium (cutoff 6000 to 8000 daltons), and each fraction was assayed for activity on resting T cells as described above in the absence or presence of added recombinant IL-2 (5 U/ml). Addition of the recombinant IL-2 was predicated on the observation that T_{HSN} enhances IL-2 responsiveness in a readily detectable fashion (legend to Fig. 2B). The profile of stimulatory activity of the column fractions in the presence of recombinant IL-2 indicates that the stimulatory activity is restricted to fractions 98 to 103 (Fig. 3). Comparison of the log of the molecular mass with the elution position of known protein molecules enabled us to assign an approximate molecular mass of 10 to 12 kD to the T_{HSN} fraction of interest. The molecular mass for human IL-2 under these conditions (open arrow) is about 20 kD, consistent with prior observations (*20*).

The [^{3}H]TdR incorporation into resting T cells generated by fractions 98 to 103 in the absence of exogenous IL-2 was less than 10 percent of the value in Fig. 3. If IL-2 by itself, or in combination with IL-1 does not (see above) induce resting T cells to proliferate, these data would imply that a major function of the 10- to 12-kD T_{HSN} factor is to induce IL-2 receptor expression within the resting T cell population. Whether the induction of IL-2 gene activation which accompanies triggering mediated by anti-$T11_2$ + anti-$T11_3$ requires a greater concentration of the T_{HSN} lymphokine than used here, or an additional cofactor absent from the 10- to 12-kD peak is not known. This issue will be resolved only when there is a larger quantity of the 10- to 12-kD fraction available.

We propose to name the 10- to 12-kD helper T cell–derived lymphokine that we describe interleukin-4A (IL-4A). This moiety is distinct from IL-2 on the basis of (i) its different apparent molecular mass; (ii) the capacity to induce IL-2 receptor expression in the resting T cell compartment; (iii) inability to be depleted from T_{HSN} with anti-IL-2 monoclonal antibody affinity chromatography (*21*); and (iv) rapid removal by absorption with the IL-2 receptor negative REX tumor. Although IL-4A, like IL-1, influences IL-2 responsiveness, other functional and molecular characteristics are distinct. For example, IL-1 cannot facilitate IL-2–mediated proliferation of T cells or thymocytes in the absence of mitogen (Table 2). Furthermore, IL-4A is a T cell product whereas IL-1 is not. In addition, IL-1 has a larger molecular mass than IL-4A (*22*).

A soluble mediator that is secreted by helper T cells after antigen specific triggering and that functions to recruit resting T cells into an activated state undoubtedly represents a major biochemical vector in causing amplification of the cellular immune response. The existence of IL-4A may provide a rational explanation for the T cell immune system's capacity to maintain millions of receptor specificities by virtue of individual Ti alpha-beta V domain combinations and still provide a protective response against virtually any nominal antigen in a rapid fashion. The possibility remains that IL-4A recruited cells may in turn themselves produce IL-4A and facilitate another level of amplification. In this amplification cascade, antigen specificity would be maintained by the specificity of the inductive event, particularly if IL-4A acts over a short distance or if IL-2 derived from the antigen-specific helper cell is rate-limiting in the IL-4A mediated response. These possibilities are not mutually exclusive. Whether other mitogenic substances are related to IL-4A remains to be determined (*23*).

There must be normal homeostatic controls to terminate the IL-4A mediated activation and amplification process. Without such controls, pathologic lymphoproliferation might ensue. Whether aberrations in such controls are in part responsible for T cell lymphoproliferative disorders is unknown. Such a possibility must be considered, particularly in view of the fact that T cell tumors induced by human T cell lymphotropic virus I (HTLV-I) express

high numbers of IL-2 receptors and a tumor cell–derived factor has been reported, like IL-4A, to induce IL-2 receptors in T-lineage cells (*24*).

The T3-Ti complex has been shown to regulate T11 pathway activation within a given population (*1*). For example, T3-Ti stimulation makes the T11 pathway refractory to triggering by anti-T11. The molecular basis for this autoregulation at the single-cell level is unknown. However, in experiments not presented here, we also found that prior incubation of resting T cells with nonmitogenic anti-T3 inhibits subsequent activation with the combination of IL-4A and IL-2. As in the case of anti-T11–mediated stimulation, this refractory period persists for as long as the T3-Ti ligand is present or up to 72 hours after its removal. Thus, the antigen-specific clone that initiates the immune response and produces IL-4A cannot respond to IL-4A itself. This mechanism, in effect, prevents the antigen-triggered cell from proliferating indefinitely to its own IL-4A. One can imagine that there must be other forms of regulation of the IL-4A recruited resting T cell population as well.

If the T11 pathway is operative throughout T-lineage ontogeny, the IL-4A may be important both for activation of thymocytes as well as peripheral T cells (*10*). Because T11+T3− NK cells demonstrate T-lineage surface antigens and RNA transcriptional phenotypes identical to those of early thymocytes (*25*), we can expect that IL-4A also serves as a T cell lymphokine to recruit natural killer cells. Whether IL-4A is involved in differentiation of thymocytes or NK cells is unknown, but appears a distinct possibility.

The similarities between effects of IL-4A for resting T cells and other recently described helper T cell factors that activate resting B lymphocytes require comment (*26*). The latter stimulate resting B cells by inducing a marked increase in surface Ia expression, causing an increase in resting B cell size, inducing G_0-G_1 transition and in some cases facilitating movement of B cells through the cycle into S phase and differentiating into Ig-secreting cells. Some structural homologies among certain of these factors and IL-4A might be anticipated, although further elucidation will require additional biochemical and molecular analysis.

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13. For generating supernatants, T cell clones were incubated in 5-ml polypropylene tubes (Falcon) at 1×10^6 cell/ml in 2.5 ml of complete medium consisting of RPMI 1640 (Gibco) containing 10 percent, heat-inactivated pooled human AB serum (Pel-Freeze) supplemented with 200 m*M* L-glutamine and 1 percent penicillin-streptomycin (Gibco) for 24 hours (unless otherwise stated) at 37°C in the presence of 25 μl of centrifuged Sepharose beads (Pharmacia, Uppsala, Sweden), to which protein A–purified anti-Ti or anti-$T4_A$ was covalently linked at 5 mg per milliliter of swollen beads (*1*). At the end of the incubation, the tubes were centrifuged; the supernatants were filtered through a 0.45-μm membrane (Millipore) and used immediately or stored at −70°C. Control supernatants were derived from parallel incubations without anti-Ti–coated Sepharose in the presence or absence of IL-2 (10 U/ml).
14. As a source of resting T cells, peripheral blood mononuclear cells were obtained by density gradient centrifugation (Ficoll-Hypaque) of peripheral blood from healthy donors. T lymphocytes were then separated by rosette formation (E+ fraction) with 5 percent sheep erythrocytes and subsequent density gradient centrifugation. Further purification was achieved by complement-mediated lysis of E+ fractions. In brief, E+ cells (2×10^7 to 3×10^7) were resuspended in 1 ml of complete medium containing a 1:100 dilution of anti-Ia, anti-T9, anti-Mo1, and antibody to IL-2 receptor and were incubated at room temperature for 30 minutes. Subsequently, 0.5 ml of selected rabbit serum (Pel-Freeze) was added as a source of complement and the cells were incubated in a water bath shaken at 37°C for an additional hour. By cytofluorographic analysis, the viable cells after the treatment were >99 percent $T3+$ and <1 percent IL-2 receptor positive and therefore are referred to as resting T cells. For proliferative assay, resting T cells (5×10^4) were incubated in triplicate cultures with 0.2 ml of a 1:3 dilution of the supernatant in round-bottomed microtiter wells (Costar) unless specifically stated otherwise. After 96 hours (or an earlier specified time) of in vitro incubation at 37°C with 6 percent CO_2 in a humidified atmosphere, cultures were labeled for 16 hours with 1 μCi of [^{3}H]TdR per well and harvested on a MASH II apparatus (Microbiological Associates). The [^{3}H]TdR incorporation was measured in a Packard liquid scintillation spectrometer (Packard Instruments, Downers Grove, IL); the mean of triplicate samples was less than <10 percent in all cases.
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16. Jurkat-derived IL-2; provided by K. A. Smith, Dartmouth Medical School, Hanover, NH.
17. Antibodies to T-lymphocyte surface antigens were derived from the following clones: 2Ad2A2 (anti-$T3_A$, IgM, mature T cells); 19ThyD7; (anti-$T4_A$, IgG_2, inducer subpopulation); 3Pt2H9 (anti-$T11_1$, IgG_1); 101D2-4C1 (anti-$T11_2$, IgG_2, thymocytes, and mature T cells); 1mono2A6 (anti-$T11_3$, IgG_3, activated T cells); 1HT4-4H3 (anti-IL-$2R_1$ receptor molecule, IgG_{2a}); anti-Ti_3 (IgG_{2a}, anti-clonotype [Ti] for 5B, AA18, AA8, and AA14 clones); 1HT4-4E5 (IgG_1, anti-Ti for the AC_3 clone); anti-Ti_4 (IgG_1, anti-clonotype of the RW17C clone); 2T8-12F10 (IgG_1, anti-Ti specific for the 20a6 clone). A monoclonal antibody specific for the monocyte-granulocyte series (Mo1, IgM) was provided by Dr. Robert Todd III, University of Michigan. These antibodies and TM11 (anti-Ia, monomorphic) and anti-T9 (antibody to transferrin receptor) have been described (*1, 2, 11, 18*).
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21. In this absorption experiment, T_{HSN} was immunoabsorbed on columns of either mouse monoclonal IgM anti-IL-2 (DMS-5) (provided by K. A. Smith) or mouse monoclonal IgM anti-T12 covalently linked to Affigel-10 (10 mg of purified antibody per milliliter of gel). In each case at 4°C, 1-ml columns were first treated with 10 ml of 0.2*N* acetic acid, neutralized with 50 ml of 10 m*M* tris-HCl, *p*H 7.4, containing 0.05 percent sodium azide. Culture supernatant (10 ml) was subsequently applied to the columns at a flow rate of 0.5 ml/min. For each, the first 1 ml of eluate was discarded and we collected the other 9 ml. We then tested the absorbed supernatants for their ability to induce proliferation of resting T cells (legend to Fig. 1). The T_{HSN} contained the same proliferative activity before or after absorption with either antibody. In a concurrent experiment, supernatant from phytohemagglutinin-stimulated peripheral blood lymphocytes was examined in a T cell clone proliferation assay which confirmed that the DMS-5 column completely removed IL-2, whereas the anti-T12 column had no effect.
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