REGULATION OF MURINE LYMPHOKINE PRODUCTION IN VIVO

III. The Lymphoid Tissue Microenvironment Exerts Regulatory Influences over T Helper Cell Function

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Cytokines, which collectively include the lymphokines and monokines, represent a family of bioactive polypeptides that are secreted in response to appropriate stimulation by a variety of both lymphoid and nonlymphoid cells. Distinct cytokines, through autocrine, paracrine, or endocrine mechanisms, are responsible for the activation and propagation of immune responses (1), as well as for coordinating the maturation of different cell lineages of the hematopoietic system (1, 2). It is therefore not unreasonable to assume that the synthesis of the various types of cytokines must be under strict regulation at all times, both at the cell and organ levels.

We recently reported that exposure of normal mice to the inflammatory effects of UV radiation (UVR),[1] bacterial LPS, or IL-1β caused a change in the responses of T cells from their peripheral lymphoid organs to antigen or mitogen stimulation (3). Activated lymphocytes from the spleen or lymph nodes of exposed donors produced markedly reduced levels of IL-2 and IFN-γ, while simultaneously secreting elevated quantities of IL-4. These findings are consistent with reported alterations in immunologic potential observed in UVR-, LPS-, or IL-1 treated animals (4-8). Analysis of the responsible mechanism(s) for this effect determined that normal animals treated with metyrapone (an 11β-hydroxylase inhibitor) (9), or RU486 (a potent glucocorticoid [GC] antagonist) (10), were refractory to inflammation-induced changes to their T cell growth factor (TCGF) production (Moon, H. B., D. S. Han, B. A. Araneo, and R. A. Daynes, manuscript submitted for publication). These findings suggested that GCs, secondarily elevated in response to IL-1-mediated effects on the hypothalamic-pituitary-adrenal axis (11, 12), were in someway responsible for altering the pattern of TCGF produced by heterogeneous populations of activated T cells in animals exposed to inflammatory stimuli. Further support for this hypothesis was derived from studies that demonstrated that enriched populations of T cells from normal donors, as well as cloned T cell lines (capable of both IL-2 and IL-4 produc-

tion after activation), secreted reduced levels of IL-2 and elevated levels of IL-4 subsequent to a direct exposure to physiologic or pharmacologic levels of GCs in vitro or in vivo (13).

Studies have now determined that dehydroepiandrosterone (DHEA), the major androgen steroid hormone in humans (14–17), is capable of enhancing the potential of murine T cells to produce IL-2 subsequent to appropriate activation (18). In addition to a direct effect on T cells in vitro, DHEA treatment of normal or GC-treated animals resulted in an enhanced capacity of their T cells to produce IL-2 in response to antigen or mitogen stimulation.

Herein, we report the results of experiments designed to question the regulatory control being exerted on T cells in vivo with regards to their capacity to produce selected lymphokines after activation. Out studies demonstrate that IL-2 represents the dominant contributor to TCGF activity by mitogen- or antigen-stimulated T cells isolated from lymphoid organs of normal mice that receive their drainage from nonmucosal tissue sites. Conversely, IL-4 was found to represent the predominant TCGF produced by resident T cells obtained from mucosal lymphoid organs, and peripheral lymph nodes that receive afferent lymphatic drainage from the various types of mucosal tissues (e.g., salivary gland, lungs, genitourinary tract, and gut). Further, our results indicate that steroid hormones, produced locally from inactive precursors, may control the functional potential possessed by T cells that reside in a given lymphoid compartment.

Materials and Methods

Mice

C3H/HeN and (C3H/HeN × BL/6)F_1 mice were bred and housed in the University of Utah Vivarium from breeding stock originally purchased from the National Cancer Institute (Bethesda, MD). Alternatively, adult C3H/HeN mice were purchased from the National Cancer Institute, isolated for a minimum of 2 wk upon arrival, and then used in experiments. Mice used for this study varied between 12 and 24 wk of age. Age-matched animals were used in each individual experiment, and the number of mice in a single experimental group varied between two and eight. The animal facility at the University of Utah guarantees strict compliance with regulations established by the Animal Welfare Act.

Antigens and Reagents

Antigens.   OVA used for immunization was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further modification or purification. The OVA was dissolved in isotonic saline at a concentration of 20 mg/ml and stored at $-20°C$ until required.

Antibodies.   Several mAb reagents were used in the present study. All mAb-containing culture supernatants were derived from hybridoma cell lines grown in our laboratory. We obtained the anti-IL-2-secreting hybridoma, S4B6, as a gift from T. Mosmann, DNAX (Palo Alto, CA). The hamster antimurine CD3 mAb-secreting hybridoma (145-2C.11.4.5) was obtained from J. Bluestone (University of Chicago). The hybridoma-producing antibody specific for murine IL-4 (11B11) was purchased from the American Type Culture Collection (Rockville, MD).

Bioactive Compounds.   The GCs and androgen steroids used in our studies were purchased from Sigma Chemical Co., except for the glucocorticosteroid antagonist, RU486, which was a gift from Dr. Murray Mitchell at the University of Utah. Each was stored as a stock solution at 10^{-2} M in ethanol, and diluted into complete media just before its addition to culture. Biodegradable corticosterone and RU486 pellets, designed to release their incorporated drug over a 20-d period, were obtained from Innovative Research (Toledo, OH).

Murine Recombinant ILs.   To provide us with a source of recombinant forms of murine IL-2

and IL-4, we obtained X63-IL-2 and X63-IL-4 transfectants from Dr. Fritz Melchers (Basel, Switzerland). These cell lines were derived from X63Ag8-653, a non-Ig-secreting murine plasmacytoma, transfected with either a 563-bp cDNA for murine IL-2 or a 500-bp fragment of the murine IL-4 cDNA (19). X63-IL-2 and X63-IL-4 lines were used to produce conditioned cell culture supernatants containing high titers of IL-2 or IL-4, which were used to maintain the growth of the IL-2/IL-4–dependent cell line CTLL-2, and to monitor the specificity of our lymphokine bioassays. Under these culture conditions, maximum sensitivity to both TCGFs was maintained, greatly facilitating the quantitation of IL-2 and IL-4 activities by bioassay.

Bioassays for IL-2 and IL-4

Based on the observation that IL-2 and IL-4, but not IL-1, IL-3, IL-5, or GMCSF, support viability of the CTLL-2 indicator cell line used in our bioassays, total TCGF activity and the relative contribution of IL-2 and IL-4 in individual test supernatants could be assessed by comparing the ability of serial dilutions of test supernatants to maintain the viability of indicator cell lines in the presence of specific blocking antibodies (20). The ability of a test supernatant (in the presence or absence of blocking antibodies) to maintain CTLL-2 viability was evaluated by the use of 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) added at a final concentration of 5 μg/ml for the final 2–4 h of a 24-h bioassay (21). DMSO was added to the culture wells to dissolve the blue formazan crystals, followed by reading the absorbance of each well at 570 nM with a Titertek Multiscan ELISA reader (Flow Laboratories, Inc., McLean, VA). Alternatively, cell viability of the indicator cell lines was evaluated microscopically (22) with the final endpoint being conservatively estimated as the highest dilution (twofold) of test supernatant allowing >50% viability of the indicator cells at 24 h. In parallel assays of test supernatants containing known quantities of IL-2 and IL-4, we have established that the spectrophotometric assay correlates well with the visual method in determining the relative contribution of IL-2 and IL-4 present in complex test supernatants. The microscopic method, however, proved to be two- to fourfold more sensitive than spectrophotometric analysis.

Immunizations and Adoptive Transfers

Mice were immunized with 100 μg of OVA emulsified in CFA. For adoptive transfers, donor lymphocytes were obtained from the peripheral lymph nodes, spleen, or Peyer's patches of OVA-primed donors (4–6 wk post-immunization). Dissociated antigen-primed lymphoid cells were adoptively transferred intravenously into naive syngeneic recipients. After a 1-wk period, recipient animals were killed and their lymphoid organs (peripheral lymph nodes, spleen, mesenteric lymph nodes, and Peyer's patches) were analyzed quantitatively for the patterns of TCGF (IL-2 and IL-4) secreted by their OVA-specific T cells.

Culture Conditions

Single cell suspensions were prepared from peripheral lymph nodes, spleens, or Peyer's patches from normal or immunized mice, and cultured at a density of 10^7 cells/ml either in 1-ml volumes into 24-well Cluster culture plates (Costar, Cambridge, MA) or in 0.2-ml volumes in 96-well microtest plates (Falcon Labware, Oxnard, CA). Culture medium consisted of RPMI 1640 supplemented with 1% Nutridoma-NS (Boehringer Mannheim Biochemicals, Indianapolis, IN), antibiotics, 200 mM L-glutamine, and 5 × 10^{-5} M 2-ME. The cultured cells were stimulated with either 100 μg/ml of OVA or a predetermined optimum concentration of anti-CD3, and then incubated for 24 h at 37°C in 10% CO_2 in a humidified incubator. Culture supernatants were harvested, clarified by centrifugation, and stored at −20°C until bioassayed for specific lymphokines.

DHEA Sulfatase Assay

Lymphoid tissues and other tissues types to be analyzed for DHEA-sulfatase activity were collected from normal mice immediately after cervical dislocation. The individual tissues were placed into a small volume (200–500 μl) of chilled lysis buffer (5 mM Tris-HCl, 2 mM EDTA, 7 mM mercaptoethanol, pH 7.5) containing 0.1% Triton X-100 and sonicated on

ice (5 s at 50 W, sonifer equipped with a microtip; Bronson Sonic Power Co., Danbury, CT). Protein determinations were made (protein assay; Bio-Rad Laboratories, Richmond, CA) on the individual homogenates using a BSA standard, and the protein concentration of each was adjusted to 1 mg/ml by the addition of ice-cold lysis buffer. A modification of the method of Ropers et al. (23), was used for determination of DHEA sulfatase activity in the tissue samples. Data given represents the mean of triplicate samples in picomole of DHEA-sulfate converted/mg of protein/h.

Results

Lymphocytes Obtained from Mucosal and Nonmucosal Lymphoid Organs of Normal Animals Produce Different Patterns of TCGF after Mitogen Activation. Lymphocytes, asceptically collected from peripheral lymph nodes (axillary, brachial, and inguinal), spleen, mesenteric lymph nodes, and Peyer's patches were stimulated with an optimum amount of anti-CD3. Cell supernatants were collected after 24 h of incubation at 37°C from triplicate cultures of each individual tissue, followed by a quantitative bioanalysis for IL-2 and IL-4. The results (Fig. 1) demonstrate that IL-2 represents the major species of TCGF produced by anti-CD3-activated lymphocytes obtained from peripheral lymph nodes and spleen, a finding that is totally consistent with previously reported observations made by other investigators (24, 25). Lymphocytes obtained from the mesenteric lymph nodes, which receive extravasating lymphocytes from both the blood and from the Peyer's patch drainage, produce approximately equal activities of IL-2 and IL-4 after in vitro activation. In contrast, lymphocytes obtained from Peyer's patches of normal mice produced IL-4 as the predominant species of TCGF after activation with anti-CD3. Identical experiments using a variety of different mouse strains and activation conditions have been repeated numerous times with equivalent results. These findings suggested the existence of regulatory mechanisms that are operative in vivo, that control the major species of TCGF capable of being produced by activated lymphocytes resident in mucosal or nonmucosal lymphoid organs. Such results might be explained from either a selectivity in the homing behavior of lymphocytes in the recirculating T cell pool (26), or preferential requirements by members of individual T cell subsets for distinct types of APC (27, 28). Alternatively, however, these results could also be explained through a conservative mechanism where quantitative and qualitative potentials for the production of selected lymphokines (e.g., TCGF) are microenvironmentally provided to individual recirculating lymphocytes during their brief residence within a particular type of lymphoid organ. The objective of the present investigation was to evaluate these possibilities and provide evidence that strongly favors the hypothesis that the types and quantities of lymphokines produced by individual activated lymphocytes are under controlling influences provided during residence within a particular lymphoid organ.

GCs and GC Antagonists are Capable of Altering the Capacity of Activated T Cells from all Lymphoid Organs to Produce IL-2 and IL-4. Groups of normal mice were subcutaneously implanted with a small biodegradable pellet containing either the GC corticosterone or the potent GC antagonist RU486. A third group of animals was left untreated as controls. The pellets release their incorporated drug at a fairly constant rate of 10 μg/h for corticosterone, or 250 ng/h for RU486 over a 2-3 wk period. 3-5 d after the initiation of treatment, animals from each of these groups were killed and the lymphocytes from their various lymphoid organs were individually collected and stimulated wtih optimum amounts of anti-CD3 in vitro. Fig. 2 provides the

normal PLN

O D (570 nm)

1 /dilution

A

normal SPLEEN

O D (570 nm)

1 /dilution

B

normal MLN

O D (570 nm)

1 /dilution

C

normal PEYER'S PATCH

O D (570 nm)

1 /dilution

D

E

normal lymphoid tissue

Peripheral LN

Spleen   512 Units

Mesenteric LN

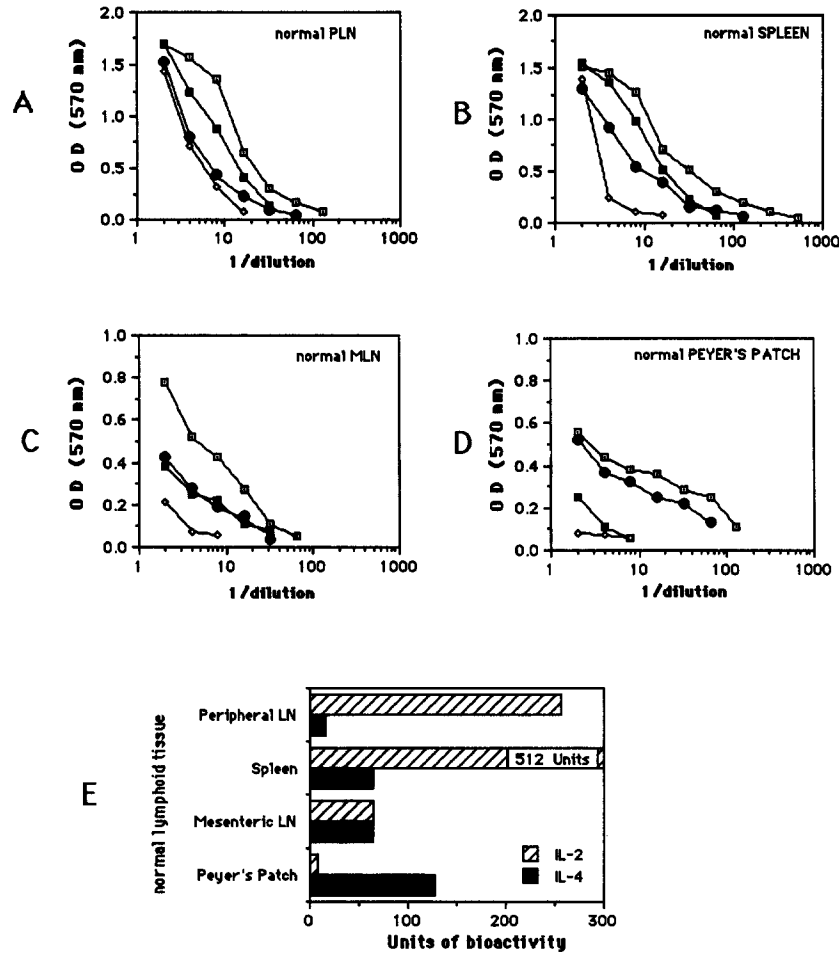Peyer's Patch

IL-2
IL-4

Units of bioactivity

FIGURE 1. Total TCGF, IL-2, and IL-4 production by lymphocytes isolated from distinct murine lymphoid tissues. Lymphocytes isolated from mucosal and nonmucosal lymphoid tissues of normal (C3H × BL/6)F₁ mice were activated in vitro with anti-CD3. 24 h later, cell culture supernatants were assayed spectrophotometrically (see Materials and Methods) for the total TCGF (no blocking antibodies; □), for the relative amount of IL-2 activity (anti-IL-4-blocking antibodies; ■), for the relative amount of IL-4 activity (anti-IL-2-blocking antibodies; ●), and for uninhibited lymphokine activity (both blocking antibodies; ◇). (A) IL-2 and IL-4 profiles of anti-CD3-activated peripheral lymph nodes (PLN), (B) spleen, (C) mesenteric lymph nodes (MLN), and (D) Peyer's patch. (E) A graphic presentation of IL-2 and IL-4 in units of bioactivity produced by anti-CD3-stimulated cultured lymphocytes from the four lymphoid tissues by visual inspection of viable indicator cells (see Materials and Methods). Note that the two assay methods reveal the identical relationship in lymphokine profiles. IL-2 represents the dominant TCGF produced by activated lymphocytes in the spleen and peripheral lymph nodes, and IL-4 represents the major TCGF produced by activated Peyer's patch lymphocytes. Similar types of comparisons between assay methods have been performed on numerous test supernatants with excellent agreement between the spectrophotometric and microscopic assays.
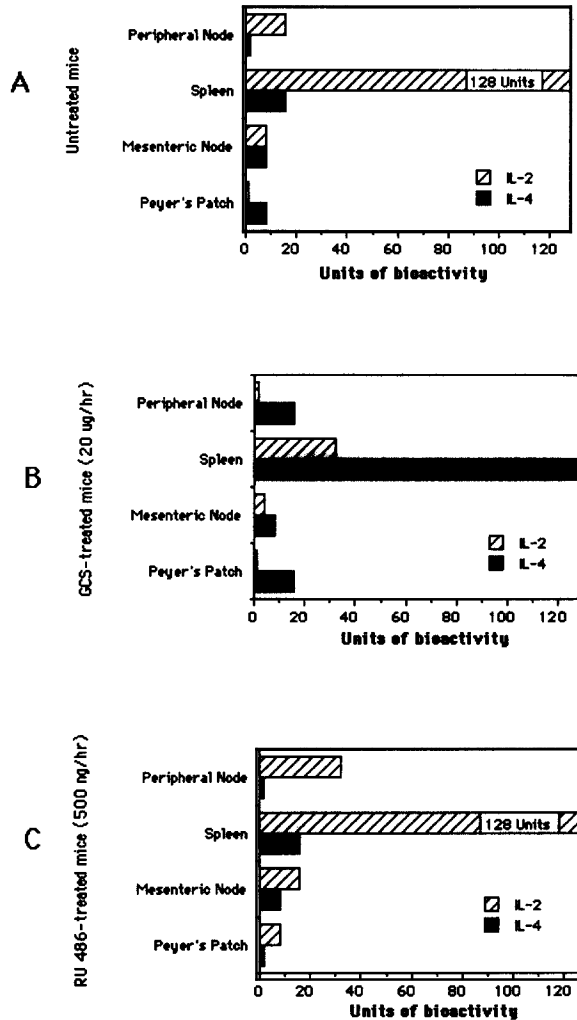
FIGURE 2. Relative IL-2 and IL-4 bioactivity produced by lymphocytes isolated from the various lymphoid organs of (A) normal C3H mice, and (B) normal mice implanted with a biodegradable pellet containing the GC hormone corticosterone 2 d before death and lymphocyte isolation, or (C) normal mice implanted with a biodegradable pellet containing the potent GC antagonist RU486. An analysis of IL-2 and IL-4 bioactivity in test supernatants was made 24 h after in vitro stimulation of lymphocytes with optimum amounts of anti-CD3. The in vivo effects of corticosterone and RU486, on subsequent lymphokine production by activated lymphocytes isolated from treated animals, has been determined in at least three different experiments, and is found to be highly reproducible.

results of a representative experiment where the supernatants from each individual culture were quantitatively analyzed for the presence of IL-2 and IL-4. The results of this experiment demonstrate that systemic elevations in GC levels in vivo can significantly alter the potential for TCGF production by lymphocytes obtained from all of the evaluated lymphoid compartments. Regardless of cell source, activated lymphocytes from GC-treated donors produced IL-4 as the predominant TCGF. Systemic treatment of normal mice with the potent GC antagonist RU486 appeared to have a limited, although highly reproducible, effect on the lymphocytes obtained from the mucosal lymphoid tissues only. The capacity of stimulated lymphocytes obtained from the mesenteric lymph nodes or Peyer's patches to produce IL-2 was consistently enhanced by a systemic RU486 treatment of the donor animals.

We recently found that a direct GC treatment of heterogeneous populations of lymphoid cells, enriched T cells, T cell hybridomas, or cloned T cell lines, before

their activation with antigens or mitogens, resulted in a marked shift in the patterns of TCGF capable of being synthesized and secreted (13). This led us to question whether similar susceptibilities to GC-mediated influences existed in lymphocytes obtained from various types of lymphoid tissues. Normal mice served as donors of peripheral lymph nodes, spleen, mesenteric lymph node, and Peyer's patch lymphocytes. Cells from each population were divided into three aliquots, and one was exposed for 30 min to 10^{-6} M corticosterone and another to 10^{-7} M RU486 before extensive washing to remove all exogenous steroid hormone. The cell cultures were then adjusted to 10^7 cells/ml and identically stimulated with anti-CD3. The results (Fig. 3) demonstrated that exposure of all lymphoid cell sources to GC in vitro resulted in a reduction in their capacity to secrete IL-2 in a concomitant increase in the production of the TCGF IL-4 (Fig. 3 *B*). The effect caused by RU486 treatment in vitro was modest, with small enhancements observed in the production of IL-2 by both the spleen and Peyer's patch lymphocytes.

Antigen-primed T Cells Switch their Potential for Producing TCGF when in Particular Lym-
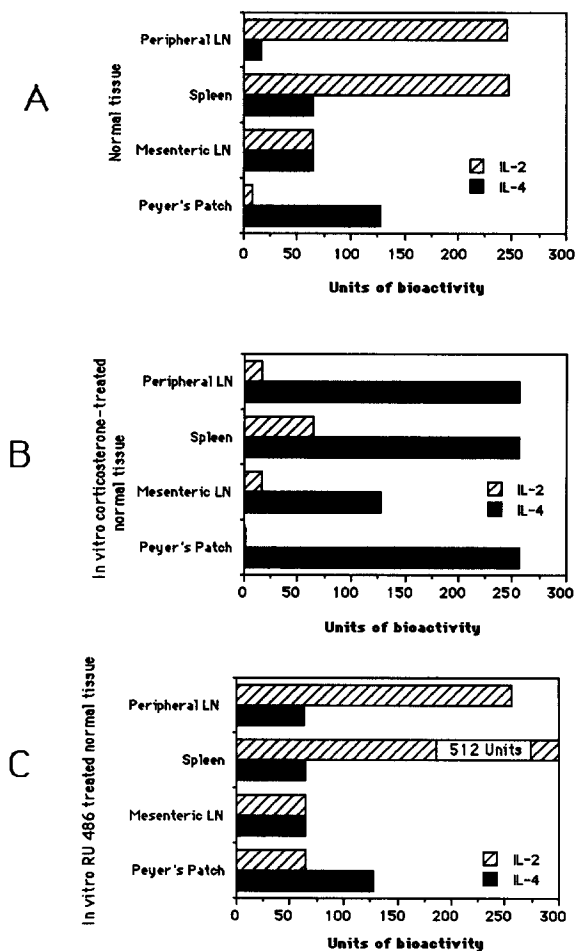

FIGURE 3. The direct effects of GCs and RU486 on the relative IL-2 and IL-4 activity measured in culture supernatants of lymphocytes derived from distinct murine lymphoid tissues. Lymphocytes isolated from mucosal and nonmucosal lymphoid tissues of normal (C3H × BL/6)F$_1$ mice were stimulated with anti-CD3. 24 h later, cell supernatants were assayed for the relative activity of IL-2 and IL-4 by visual inspection of the indicator cell line (see Materials and Methods). (*A*) Anti-CD3 activation of lymphocyte populations obtained from normal mice and (*B*) lymphocytes isolated from normal mice cocultured with anti-CD3 after a 30-min pulse with corticosterone (10^{-6} M). (*C*) Anti-CD3-activated lymphoid cells from normal mice where cells were exposed to a 30 min pulse of 10^{-7} M RU486. The IL-2 and IL-4 profiles of activated lymphocytes from the different lymphoid tissues have been assessed numerous times with equivalent results.
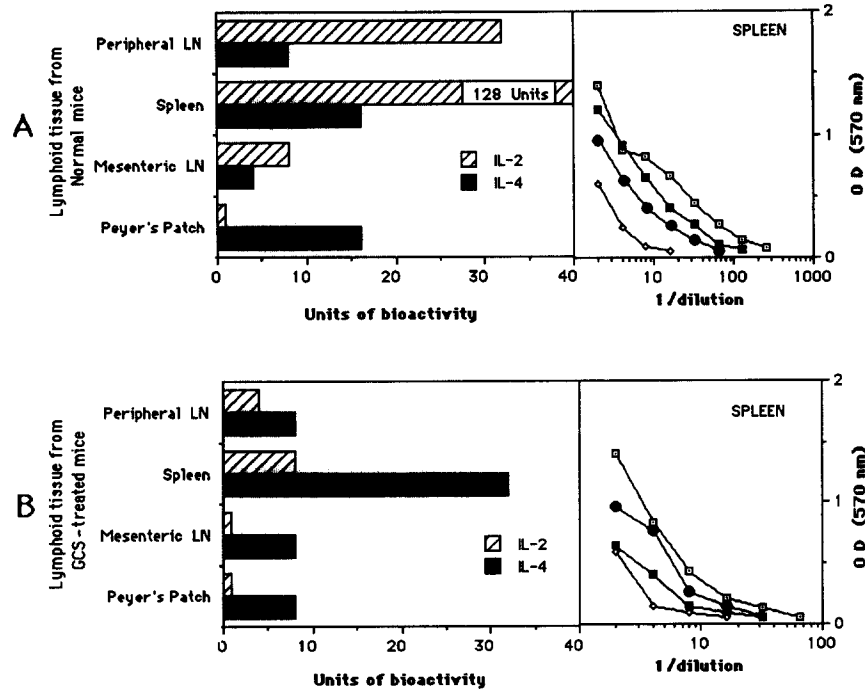
FIGURE 4.   The secretion of IL-2 and IL-4 by OVA-primed lymphocytes isolated from various lymphoid organs after antigen activation in vitro. Groups of C3H mice were immunized 3 wk previously with OVA. (A) The bioactivities of IL-2 and IL-4 produced by OVA-stimulated lymphocytes from the lymphoid tissues of normal mice with a comparison between the spectrophotometric and the microscopic methods for spleen. (B) The bioactivities of IL-2 and IL-4 produced by OVA-stimulated lymphocytes from animals implanted with a biodegradable corticosterone pellet (designed to release GCs at a rate of ∼12.5 μg/d) 5 d before immunization. A comparison between spectrophotometric and microscopic methods for the spleen is also provided (see Fig. 1). This specific analysis of antigen-induced IL-2 and IL-4 production by the various lymphoid tissues has been performed twice with excellent agreement in experimental results between studies.

phoid Organs.   Normal mice were immunized with the antigen OVA, and their lymphoid organs removed after 3 wk for analysis of antigen-induced TCGF production. The results of these assays (Fig. 4 A) determined that the relationship between IL-2 and IL-4 activity produced by isolated lymphocytes in response to antigen was comparable with that observed after an anti-CD3 stimulation of similar tissues isolated from normal mice (e.g., IL-2 represented the dominant TCGF activity produced by peripheral lymphoid tissues and IL-4 was the predominant Peyer's patch TCGF). Analysis of the IL-2 and IL-4 titers produced by OVA-activated T cells, isolated from animals that were immunized with antigen while under the systemic influence of GCs, further established that IL-4 now represented the predominant TCGF produced from all lymphoid compartments (Fig. 4 B). Collectively, these results are consistent with the hypothesis that while the specificity of antigen responsiveness by Th cells is regulated through specific recognition by the TCR, the capacity of these cells to produce lymphokines (at least TCGF, IL-2, and IL-4) may be controlled through influences provided within a given tissue microenvironment. Fur-

ther, these inherent influences appear to be susceptible to change by systemic endocrine effects caused directly by elevations in plasma levels of GC hormones (effects that appear to diminish the production of IL-2, and augment the production of IL-4 [13]).

Our results, so far, failed to discount the possibility that the differences we observed in TCGF production were being mediated through the selective recruitment of T cell subsets into mucosal or nonmucosal lymphoid organs, with distinct subsets having a precommitted potential for a dominance in IL-2 or IL-4 production after cellular activation. In an attempt to question this possibility, peripheral lymph node lymphocytes, splenic lymphocytes, and Peyer's patch lymphocytes were collected from a group of OVA-primed donors 3 wk after their immunization with antigen. Stimulation of an aliquot from each of these isolated cell populations with OVA in vitro determined that their capacity to produce IL-2 and IL-4 was similar to that presented in Fig. 4, where IL-2 dominated the TCGF activity in supernatants from peripheral lymph nodes and spleen, and IL-4 dominated the TCGF activity from the Peyer's patch lymphocytes (data not shown). Each of these cell populations was adoptively transferred into individual groups of syngeneic normal recipients, and the adoptive recipients rested for a period of 1 wk before death for analysis of their lymphoid compartments for TCGF production in response to in vitro OVA stimulation. The results of this experiment (Fig. 5) determined that antigen-primed lymphocytes from all three donor tissues (lymph node, spleen, and Peyer's patch) could gain access into both the mucosal and nonmucosal lymphoid tissues of normal recipients, and that the pattern of TCGF production by the lymphocytes obtained from any given tissue appeared to be under influences being exerted by the lymphoid compartment of the recipient, not the donor. These results suggest that the preferential synthesis and secretion of either IL-2 or IL-4 by antigen-activated T cells obtained from primed donors does not appear to be due to a precommitment in functional potential being exhibited by the adoptively transferred donor cells. Rather, it appears that the mucosal lymphoid environment is highly conducive to T cell production of IL-4. Conversely, recirculating T cells that have gained temporary residence in peripheral lymphoid organs were apparently provided with environmental influences that enhance their ability to produce IL-2 after response to antigen (or mitogen) activation.

The Normal Androgen Steroid DHEA Enhances the Potential of Normal T Cells to Produce IL-2. We recently demonstrated that the adrenal androgen steroid hormone DHEA could exert direct effects on purified T cells, T cell hybridomas, and antigen-specific T cell clones (18). These effects were manifest as a marked increase in the capacity of activated T cells to produce IL-2, plus the ability of DHEA to overcome the suppressive influences of GCs on the production of this TCGF. DHEA sulfate was without an effect in these in vitro systems (18).

To evaluate the possible role(s) played by this androgen steroid in vivo, normal mice were injected intraperitoneally with either 200 μg of DHEA or 200 μg of DHEAS 3–7 d before death. The peripheral lymph nodes (axillary, brachial, and inguinal), spleen, and Peyer's patches were collected from the two experimental groups, plus from a group of normal untreated controls. Each individual cell population was stimulated with an optimum amount of anti-CD3, and the supernatants were analyzed for IL-2 and IL-4 after 24 h. The results of a representative experiment (Fig. 6) demonstrates that both DHEA and DHEA-sulfatase (when administered in vivo)
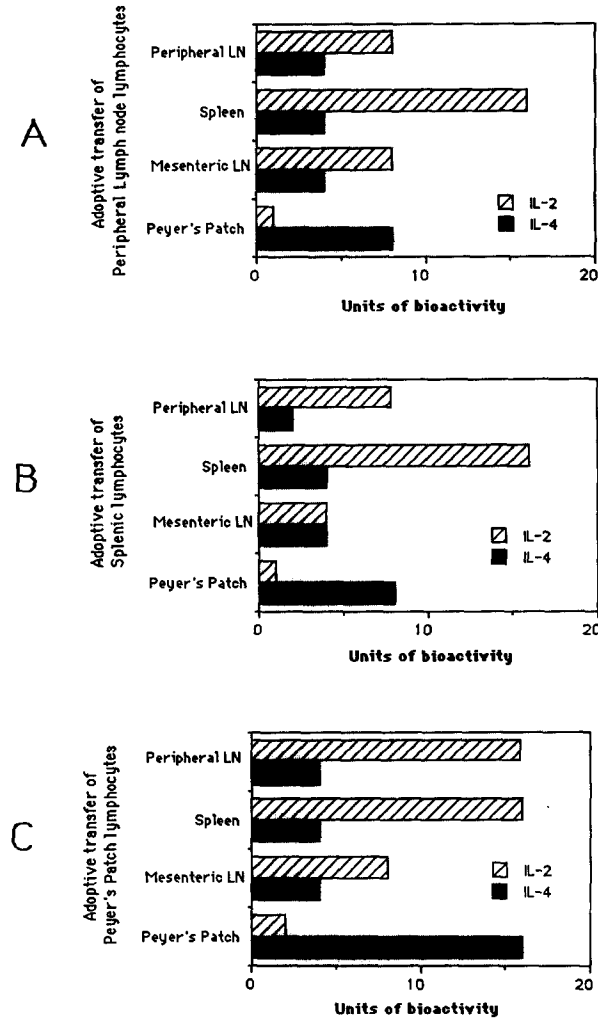
FIGURE 5. OVA-induced IL-2 and IL-4 TCGF production by lymphocytes isolated from mucosal and nonmucosal lymphoid organs of (C3H × BL6)F₁ adoptive recipients of 5 × 10⁷ (*A*) peripheral lymph node lymphocytes, (*B*) spleen, or (*C*) Peyer's patch lymphocytes obtained from syngeneic OVA-primed donors. The adoptive recipients were rested for 1 wk to allow the "memory" T cells to fully equilibrate within the recirculating T cell pool, before death, isolation of their lymphoid organs, and in vitro stimulation of their lymphoid cells to produce IL-2 and/or IL-4 in response to OVA. While parts of this experiment have been reproduced several times, the experiment, as presented in this figure, has been performed twice with equivalent results.

were capable of enhancing the ability of lymphocytes isolated from either the peripheral lymph nodes or spleen to produce IL-2 after activation. Only DHEA, however, was able to enhance the production of IL-2 by cells isolated from the Peyer's patches of treated animals. This consistent finding suggested that different lymphoid organs might contain varying levels of the enzyme DHEA-sulfatase, assuming DHEA represents the active metabolite. When such studies were performed (data not shown), we found that DHEA-sulfatase activity (pmol DHEA produced/mg protein/h) was high in the spleen and lymph nodes and low in Peyer's patches (60–80% reduced).

   The Ability of T Cells to Produce IL-2 or IL-4 after Stimulation is Directly Influenced by the Lymphoid Tissue from which they Were Isolated and Correlates with their DHEA-Sulfatase Content.   Our final experiments were designed to thoroughly question the relationship between the type of tissue (mucosal versus nonmucosal) that provides drainage to a particular lymphoid organ, and the ability of lymphocytes collected from that
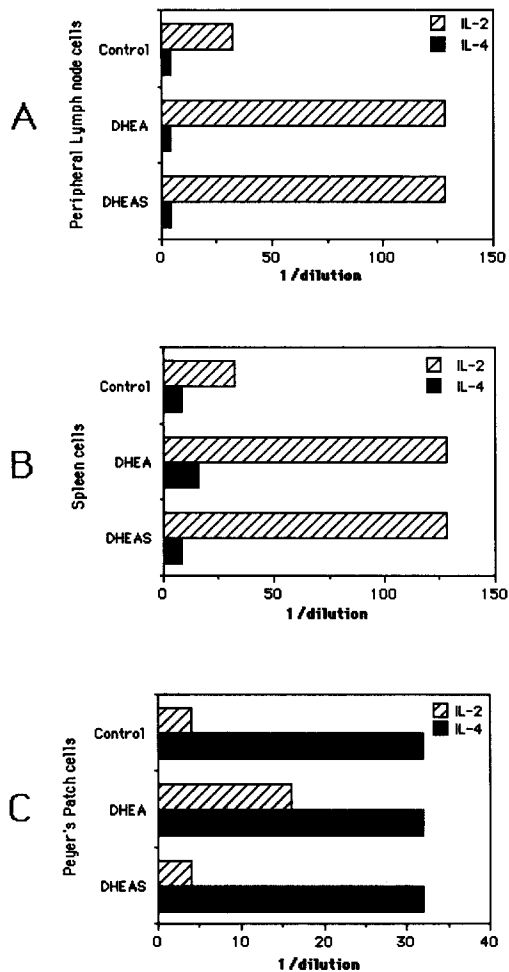
Figure 6. Lymphokine response by peripheral lymph node lymphocytes (A), splenic lymphocytes (B), or Peyer's patch lymphocytes (C) derived from normal DHEA- or DHEAS-treated animals. Normal C3H donors were given 200 μg of either DHEA or DHEAS by intraperitoneal injection 3 d before preparation of their indicated lymphoid cells for culture. Standard culture conditions, using optimum amounts of anti-CD3 for stimulation, are as described in Materials and Methods. This finding concurs with previous experiments performed by this laboratory on the effects of DHEA and DHEAS on IL-2 production when the steroid hormones are administered in vivo (18).

lymphoid organ to produce TCGF after activation. In addition, each of the test tissues was also simultaneously evaluated for DHEA-sulfatase enzymatic activity.

Groups of normal mice served as donors of cervical, axillary, brachial, parathymic, periaortic, inguinal, and mesenteric lymph nodes, spleen, and Peyer's patches. Each tissue type was individually collected, and dissociated tissues were analyzed for the capacity to secrete IL-2 and IL-4 after activation, or for DHEA-sulfatase activity. The results of these studies are presented in Table I, and demonstrate that the quantitative relationship between the activities of IL-2 and IL-4 produced by lymphocytes in response to polyclonal T cell stimulation is directly related to the tissue (mucosal vs. nonmucosal) that provides drainage to the lymphoid organ being evaluated. Lymphocytes isolated from lymphoid organs receiving their afferent lymphatic drainage from mucosal tissues (e.g., the cervical, parathymic, and periaortic lymph nodes, and the Peyer's patches) predominantly produced IL-4 after the activation in vitro. Conversely, lymphoid tissues receiving their drainage predominantly or exclusively from nonmucosal sites (e.g., the axillary, brachial, and inguinal lymph nodes and

TABLE I

Relationship between Inducible TCGF Production by Lymphocytes Derived from
Various Murine Lymphoid Organs to their Expression of DHEA-Sulfatase Activity

Lymphoid tissue	Major tissue drainage	IL-2/IL-4*	DHEA sulfatase activity[‡]
		U/ml	pmol DHEA/ms protein/h
Cervical lymph node	Mucosal	1:32 (4:128)	52
Axillary lymph node	Nonmucosal	8:1 (128:16)	294
Brachial lymph node	Nonmucosal	16:1 (256:16)	310
Parathymic lymph node	Mucosal	1:4 (4:16)	107
Periaortic lymph node	Mucosal	1:16 (4:64)	112
Spleen	Nonmucosal	4:1 (128:32)	170
Inguinal lymph node	Nonmucosal	8:1 (128:16)	190
Mesenteric lymph node	Mucosal + nonmucosal	2:1 (64:32)	134
Peyer's patch	Mucosal	1:16 (1:16)	47

* Assay for IL-2 and IL-4 used the microscopic endpoint analysis. See Materials and Methods.
‡ DHEA-sulfatase activity was analyzed on homogenates of whole tissues. Additional enzyme assays have determined that removal of erythrocytes from the spleen tissue by 0.83% ammonium chloride treatment results in DHEA-sulfatase levels that are 1.7–2 times greater than reported here.

spleen), predominantly produced IL-2 after activation. In addition, the ability of lymphocytes obtained from a particular tissue to produce the TCGF IL-2 appeared to be directly related to the amount of DHEA-sulfatase activity present within the particular tissue. Recent studies using purified lymphoid cell populations have determined that macrophages are the major contributors to the DHEA sulfatase activity (data not shown).

Discussion

Recognition of peptide antigens in the context of developmentally selected self MHC molecules, and the activation of antigen-specific clones of T cells, represent essential components in the afferent phase of an adaptive immunologic response. Establishing protective immunity to infectious agents or tumors, however, possesses a number of additional requirements that extend far beyond the mechanisms of antigen recognition and lymphocyte activation. Recent investigations have determined that the pattern of distinct species of lymphokines, which are synthesized and secreted by activated T cells, may play a pivotal role in directing the development of distinct types of immune effector responses (3). Logically, therefore, any mechanisms or influences that function to regulate the pattern of lymphokines that are produced by antigen-activated T cells would have important consequences on the types of immune responses initiated.

In animals, the majority of immunologic responses are initiated within the anatomical confines of a particular secondary lymphoid organ. It is accepted that antigen-reactive lymphocytes are activated in lymphoid organs in response to free antigen or accessory cell–associated antigen peptides that enter the organ through either afferent lymphatic drainage (e.g., lymph nodes), across gut mucosa (e.g., Peyer's patches), or directly from the blood (e.g., spleen). The secondary lymphoid organs of mammals are fixed, widely distributed throughout the body, and are unique from

one another. This uniqueness is supported both anatomically and histologically, and is probably related to the different functions that the various types of lymphoid organs are believed to serve in the context of the overall immune system. Most evident are the distinct types of immunologic responses that are necessary to provide immunologic protection to either mucosal or nonmucosal tissue sites. Therefore, in spite of the knowledge that the vast majority of mature T cells are in the recirculating T cell pool and are fully capable of extravasation or entry into all types of secondary lymphoid organs (26), the selection of one form of immunologic response over another appears to exhibit a fair degree of anatomic compartmentalization.

The present study sought to question the possible consequences associated with T cell compartmentalization into distinct types of secondary lymphoid organs with regards to the types of TCGFs capable of being produced after antigen or mitogen stimulation. We reproducibly found that antigen- or mitogen-stimulated T cells isolated from peripheral lymph nodes (axillary, brachial, inguinal) or the spleen secreted IL-2 as the dominant TCGF, while activated T cells, isolated from the Peyer's patches of normal donors, produced IL-4 almost exclusively. Systemic treatment of normal mice with GCs, or potent GC antagonists, qualitatively altered the species of TCGF capable of being produced by activated lymphocytes obtained from both mucosal and nonmucosal lymphoid organs. GC hormone treatment resulted in IL-4 representing the dominant species of secreted TCGF from all lymphoid organs, while treatment of mice with RU486 resulted in IL-2 secretion being dominant in all tested organs after activation. We also observed that a direct exposure of normal lymphoid cells to the influences of GCs or GC antagonists in vitro was capable of altering their potential to produce TCGF. These observations provided additional support for the concept that steroid hormones are able to exert both qualitative and quantitative influences over the ability of T cells to produce certain lymphokines, and reduced the possibility that tissue selective homing of T cells was responsible for the observed differences in TCGF production found in various lymphoid compartments.

Determining that the adoptive transfer of either antigen-primed peripheral lymph node lymphocytes (IL-2 dominant), splenic lymphocytes (IL-2 dominant), or Peyer's patch lymphocytes (IL-4 dominant) into normal syngeneic recipients resulted in tissue-associated TCGF responses in the recipients that were identical to those observed in the original donors, provided strong suggestive evidence that the lymphoid organs themselves were exerting some regulatory influence over the pattern of TCGFs capable of being produced by their resident T cells.

Our recent work with the normal androgen steroid DHEA established that this hormone had a strong positive influence on the ability of T cells to produce IL-2 after activation (18). DHEA represents the major adrenal steroid produced by humans between their second and fifth decades of life (14, 16), and is uniquely sulfated to DHEA-sulfate before secretion into the plasma (29). Unlike DHEA, DHEA-sulfate is incapable of affecting TCGF production by lymphocytes directly exposed to the hormone in vitro (18). Interestingly, DHEA was very effective in vivo in augmenting the capacity of isolated peripheral lymph node, spleen, and Peyer's patch lymphocytes to produce IL-2 after activation, while DHEA-sulfate was found to only be active on peripheral lymph node and spleen lymphocytes (Fig. 6). This reproducible observation suggested that DHEA represented the active hormone metabolite and that the desulfation of DHEA-sulfate to DHEA might display a level of tissue specificity.

Various murine tissues have been quantitatively analyzed for DHEA sulfatase activity (30), and lymph nodes and spleen have been described as tissues having high levels of this enzyme. No reports exist concerning the DHEA-sulfatase activity of murine Peyer's patches.

By undertaking a major comprehensive study on the secondary lymphoid organs of normal mice, we were able to experimentally support our working hypothesis of a lymphoid tissue control over T cell lymphokine production after activation. The results of these studies determined that all of the isolated lymphoid organs that are known to receive drainage from nonmucosal tissues contain T cells that predominantly produce IL-2 after activation. Conversely, all lymphoid organs that receive their drainage from mucosal tissue sites were found to produce IL-4 as the predominant TCGF (Table I). These results were paralleled by quantitative enzymatic analyses of the DHEA-sulfatase activity present within the various types of murine lymphoid tissues. We established that a direct correlation existed between the tissue DHEA-sulfatase content and the presence of an IL-2 dominance to TCGF production by activated lymphocytes from a particular organ. Such a correlation provided strong suggestive evidence that DHEA might be responsible in vivo for providing recirculating T cells that are temporarily localized within lymphoid organs drained by nonmucosal tissues, with the ability to produce IL-2 as their dominant TCGF. We envision that adrenal DHEA-sulfate is being locally metabolized to DHEA by tissue-associated DHEA-sulfatase. Paracrine effects of DHEA on tissue localized T cells would provide them with an enhanced potential to secrete IL-2 when activated. Lymphoid tissues having sufficiently high levels of DHEA-sulfatase activity would, therefore, represent the microenvironments most conducive to T cell IL-2 synthesis.

Lymphoid tissues that are known to receive drainage from mucosal tissue sites were found to possess the lowest DHEA-sulfatase activity, and T cells from these lymphoid organs produced IL-4 as the dominant TCGF. Whether the lack of sufficient DHEA-sulfatase activity in these lymphoid organs is, itself, responsible for the reproducible observation of IL-4 dominance in TCGF production is presently unknown. It is important to state that DHEA-sulfatase levels, and not the expression of peripheral lymph node- (31) or Peyer's patch–specific (32) homing receptors, served as an accurate indicator of whether IL-4 or IL-2 would dominate TCGF production (data not shown).

It is conceivable that the opposing hormonal influences of DHEA and GCs on T cells could account for the wide variations in potential for TCGF production observed in the various types of lymphoid organs. Alternatively, and representing a mechanism that we presently favor, there may exist another species of hormone that is capable of exerting a paracrine influence, predominantly on T cells temporarily localized within lymphoid organs being drained by mucosal tissues. We believe that 1,25-dihydroxyvitamin D3 [1,25(OH)$_2$D$_3$] may represent such a hormone. 1,25-(OH)$_2$D$_3$ is the active hormone for which intracellular receptors are known to exist in T cells (33, 34). It is a metabolite of 25-hydroxyvitamin D$_3$, and can be produced locally by the action of 1α-hydroxylase (an enzyme that is inducible in LPS- or IFN-γ-stimulated macrophages) (35, 36). 1,25(OH)$_2$D$_3$ has already been reported to inhibit T cell production of IL-2 and IFN-γ (37, 38), and we have recently determined that this steroid hormone is a potent enhancer of IL-4 production by activated T cells (Araneo, B. A., and R. A. Daynes, unpublished observations).

Our present research has provided us with the information necessary to develop a model to explain the differences that exist in individual lymphoid organs (related to the potential possessed by their recirculating T cells for TCGF production if activated). Our observations suggest that the vast majority of recirculating T cells possess the genetic potential to produce both IL-2 and IL-4 after activation. Transient controlling influences on TCGF production are epigenetically provided to individual T cells during their short residence within a particular lymphoid tissue. These influences appear to come from distinct species of steroid hormones, produced locally from inactive circulating precursors and acting in a paracrine manner. Steroid hormones are well known for their ability to function as transcriptional regulators of gene activity, with individual species being capable of exerting both enhancing and repressing influences on particular cellular genes (39). Our model implies that individual recirculating T cells continually undergo phase variations, in potential for lymphokine production, and are dependent upon microenvironmentally supplied influences. T cells that are resident within lymphoid organs drained by nonmucosal tissues would be in one phase where IL-2 would represent the predominant TCGF produced after activation. Conversely, T cells in transit through lymphoid organs drained by mucosal tissues would be placed in another phase, and IL-4 would predominate TCGF production. How many other T cell lymphokines are susceptible to this type of regulation is presently unknown. However, preliminary studies indicate that the regulation of IFN-γ exhibits similarities to the control of IL-2 by steroid hormones. Further, IL-4 and IL-5 production also appears to be coordinately regulated by influences provided by steroid hormones (Araneo, B. A., and R. A. Daynes, unpublished observations). Finally, our model possesses an additional important component. We hypothesize that the macrophage, already known to enter lymphoid organs predominantly through the afferent lymphatic drainage from the tissue sites (40), may play an extremely important immunologic role beyond antigen uptake, processing, and presentation. It is quite possible that the macrophage, as a consequence of inductive influences provided during its surveillance of peripheral or mucosal tissue sites, actually instructs the antigen-responsive T cell to produce specific species of lymphokines after its entrance into the draining lymphoid organ and its interaction with an antigen-responsive T cell. The actual instruction would come from epigenetically facilitated transcriptional or post-transcriptional regulatory influences exerted by unique species of steroid hormones present within the lymphoid tissue microenvironment. These functional steroid hormones would be produced locally, or enter through the afferent lymphatic drainage from inactive precursors metabolized by macrophage-associated enzymes.

Summary

We investigated the capacity of murine T lymphocytes, isolated from various lymphoid organs of normal or antigen-primed donors, to produce IL-2 or IL-4 after activation with anti-CD3 or specific antigen. Our results established that T cells resident within lymphoid organs being drained by nonmucosal tissue sites (e.g., axillary, inguinal, brachial lymph nodes, or spleen) produced IL-2 as the predominant T cell growth factor (TCGF) after activation. Conversely, activated T cells from lymphoid organs being drained by mucosal tissues (Peyer's patches, and cervical, periaortic, and parathymic lymph nodes) produced IL-4 as the major species of TCGF.

Analysis of the lymphoid tissues obtained from adoptive recipients of antigen-primed lymphocytes provided by syngeneic donors provided evidence that direct influences were being exerted on T cells during their residence within defined lymphoid compartments. These lymphoid tissue influences appeared to be responsible for altering the potential of resident T cells to produce distinct species of TCGF. Steroid hormones, known transcriptional enhancers and repressors of specific cellular genes, were implicated in the controlling mechanisms over TCGF production. Glucocorticoids (GCs) were found to exert a systemic effect on all recirculating T cells, evidenced by a marked dominance in IL-4 production by T cells obtained from all lymphoid organs of GC-treated mice, or after a direct exposure of normal lymphoid cells to GCs in vitro before cellular activation with T cell mitogens. Further, the androgen steroid DHEA appeared to be responsible for providing an epigenetic influence to T cells trafficking through peripheral lymphoid organs. This steroid influence resulted in an enhanced potential for IL-2 secretion after activation. Anatomic compartmentalization of the DHEA-facilitated influence appears to be mediated by differential levels of DHEA-sulfatase in lymphoid tissues. DHEA-sulfatase is an enzyme capable of converting DHEA-sulfate (inactive) to the active hormone DHEA. We find very high activities of this enzyme isolated in murine macrophages. The implications of our findings to immunobiology are very great, and indicate that T cells, while clonally restricted for antigen peptide recognition, also appear to exhibit an extreme flexibility with regards to the species of lymphokines they produce after activation.

Regulation of this highly conservative mechanism appears to be partially, if not exclusively, controlled by cellular influences being exerted by distinct species of steroid hormones, supplied in an endocrine or a paracrine manner where they mediate either systemic or tissue-localized influences, respectively. Our results also suggest that macrophages, known to primarily enter peripheral lymphoid organs through afferent lymphatic drainage, might be responsible for both antigen presentation, as well as being major contributors to the controlling influences on a T cell's capacity for lymphokine production.

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