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Sequential stages of human T lymphocyte differentiation

(prothymocyte/T cell markers/T cell functions/thymic factors)

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ABSTRACT **Induction of thymus-dependent lymphocyte (T cell) differentiation was performed *in vitro* with thymic factors as inducers. T cell precursors from human bone marrow first expressed surface differentiation antigens and then acquired the capacity to form rosettes with sheep erythrocytes. The latter marker could not be induced when cells with differentiation antigens had been eliminated. The proliferative responses to phytomitogens or to allogeneic stimuli appeared to be characteristics of later stages in differentiation that also can be induced or amplified by *in vitro* incubation of marrow cells or thymocytes with thymic factors. When phytomitogen-responsive cells from peripheral blood were inactivated *in vitro*, the allogeneic response was enhanced. Although these responses are acquired almost concomitantly, they are therefore envisioned to be characteristics of separate T cell subsets. After immunological reconstitution of patients, the T cell development *in vivo* involves a succession of differentiation events similar to that described above. Our experiments with mice, using similar methods, have also shown that graft-versus-host inducing capacity is a function of a cell population distinct from that which yields a proliferative response to *in vitro* stimulation by phytohemagglutinin. These results support our model of sequential differentiation of human prothymocytes into various subsets of mature T cells.**

The study of thymus-dependent lymphocytes (T cells) in various states of maturation may be regarded as an approach to a better understanding of cell differentiation in general. Recently defined surface markers and functional properties of T cells can be interpreted to be characteristics of subsets representing various differentiation states. *In vitro* induction of several of these characteristics onto T cell precursors, or prothymocytes, from mouse (1, 2) and man (3) is now feasible. We present here evidence to indicate that the following markers and functions appear successively in the development of human T cells: human T lymphocyte differentiation antigens (HTLA), capacity to form rosettes with sheep erythrocytes (E rosettes), proliferative responses to stimulation by allogeneic cells or by mitogens such as concanavalin A (Con A) and phytohemagglutinin (PHA). On the basis of evidence derived from immunological reconstitution by marrow or thymus transplantation in patients with immunodeficiencies and from *in vitro* induction of prothymocyte responses with thymic factors, proliferative responses to allogeneic and mitogenic stimulation appear to develop concomitantly. By using ablative techniques *in vitro*, it has been possible to demonstrate that different cell subpopulations are responsible for the mitogen and the allogeneic proliferative responses. T cell subpopulations thus appear to develop sequentially along diverging differentiation pathways. The definition of recognizable characteristics of several successive stages of T cell differentiation in man may find useful applications in the study of immunodeficiency diseases and immunologically mediated disorders.

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MATERIALS AND METHODS

Human peripheral blood lymphocytes were separated from heparinized venous blood by centrifugation in a Ficoll-Hypaque gradient (4). Human bone marrow cells were separated in five layers by centrifugation on a discontinuous bovine serum albumin density gradient as described (5), and thymocytes were prepared from thymuses of infants undergoing cardiac surgery.

Human spleen or thymus extracts were prepared according to the method of Goldstein (6). When thymocytes or bone marrow cells were incubated with these extracts, fraction 3 was used at a protein concentration of 500 μg/ml and fraction 5 at 250 μg/ml.

The anti-HTLA serum, the cytotoxic assay, the E-rosette formation, the induction assay, the lymphocyte cultures with mitogens or mitomycin-treated allogeneic cells, and the treatment with 5-bromodeoxyuridine (BrdUrd) and light have been described (3, 7, 8). Separation of E-rosette-forming cells (ERFC) from non-ERFC was performed by differential centrifugation, repeated twice.

Treatments for immunological reconstitution of patients with immunodeficiencies have been described in an infant with a partial DiGeorge's syndrome treated by fetal thymus transplantation (9) and in infants with severe combined immunodeficiency disease treated successfully by compatible bone marrow transplantation (10).

RESULTS

In experiments using anti-HTLA serum, we found HTLA on virtually all thymocytes and peripheral blood T cells in man. Absorption studies demonstrated that HTLA were present at a higher concentration on the surface of the former than the latter (8). The HTLA$^+$ phenotype was also shown to be readily inducible by a short (2 hr) incubation *in vitro* of bone marrow prothymocytes with factors of thymic origin (3). No further induction was observed when the period of incubation was extended to 6 hr (Table 1). HTLA can thus be considered as an early characteristic of T cell differentiation, persisting throughout the life of the cell but with reduced expression on mature T cells.

In human bone marrow, the percentage of cells forming E

Abbreviations: T cells, thymus-dependent lymphocytes; HTLA, human T lymphocyte differentiation antigens; E rosettes, rosettes with sheep erythrocytes; Con A, concanavalin A; PHA, phytohemagglutinin; BrdUrd, 5-bromodeoxyuridine; ERFC, E-rosette-forming cells; MLR, mixed leukocyte reaction; SRBC, sheep erythrocytes; GvHR, graft-vs-host reaction.

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Table 1. Differentiation of human bone marrow cells into HTLA$^+$ and ERFC$^+$ lymphocytes and effect of depletion of HTLA$^+$ lymphocytes on induction of ERFC

Preincubation		% of bone marrow cells from layer III* with characteristics		
Time, hr	Treatment	HTLA$^+$	ERFC$^+$	ERFC$^+$ (HTLA depleted)†
2	Medium	4	2	<1
	Spleen fraction 3	10	3	1
	Thymus fraction 3	26	6	<1
6	Medium	6	3	<1
	Spleen fraction 3	9	4	1
	Thymus fraction 3	27	18	1

* Bone marrow cells were separated into five layers by centrifugation on a discontinuous bovine serum albumin density gradient (5). Cells from layer III (density 1.055–1.060 g/ml) were incubated with RPMI 1640 medium as a control or with fraction 3 of human spleen or thymus extract. Cells were then washed and examined for the presence of HTLA$^+$ or ERFC$^+$ characteristics.

† Bone marrow cells from layer III were treated with anti-HTLA serum and complement. After elimination of HTLA$^+$ cells, the remaining cells were incubated with the various extracts. The percentage of ERFC was then determined.

rosettes was found to be lower than the percentage of HTLA$^+$ cells. More importantly, the 2-hr induction assay was associated with only a minimal increase in the number of ERFC (Table 1). When the period of incubation with thymic factors was prolonged to 6 hr, the percentage of ERFC increased but never reached as high a percentage as the HTLA$^+$ cells. In experiments involving the elimination of HTLA$^+$ marrow cells followed by incubation of the remaining cells with thymic factors, no induction of ERFC was possible. This finding suggests that, under normal conditions, lymphocytes with the capacity to form E rosettes derive from cells that have already acquired the HTLA$^+$ phenotype. Based upon a comparison of sensitivity to anti-HTLA serum and the ability to form E rosettes, all peripheral blood lymphocytes that formed E rosettes had surface HTLA. However, there seemed to be a few cells with HTLA$^+$ phenotype but no significant affinity for sheep erythrocytes, as shown by the surface analysis of cells remaining after removal of all ERFC (Table 2).

Table 2. Effect of complete removal of HTLA$^+$ lymphocytes or ERFC on various characteristics of human peripheral blood lymphocytes

Pretreatment	Percent HTLA$^+$	Percent ERFC	Proliferative response, cpm × 10^{-3}: 0	Con A	PHA	Allogeneic cells
C*	72	70	2	311	293	38
Anti-HTLA + C*	—	<1	4	7	8	5
Removal of ERFC†	3	<1	5	13	11	6

* Peripheral blood lymphocytes were incubated for 2 hr at 37° with complement (C) alone as a control or with anti-HTLA (dilution 1:10) + C. Cells were washed and studied for their markers and proliferative responses to stimulants *in vitro*. The proliferative response was measured by the incorporation of [^{3}H]thymidine and is expressed as cpm (mean of five experiments).

† Removal of ERFC by differential centrifugation was repeated twice and the remaining lymphocytes were studied for markers and proliferative responses.

Table 3. Amplification of Con A responsiveness of human thymocytes by pretreatment with thymic factors

Pretreatment of thymocytes	Dose of Con A, μg/ml	Proliferative response, cpm × 10^{-3}
Control medium	0	1.0
	20	4.0
	40	4.0
	80	5.0
	160	2.0
Human thymus fraction 5	0	1.5
	20	1.5
	40	20.0
	80	19.5
	160	2.0

Thymocytes were incubated for 18 hr at 37° with RPMI 1640 medium as a control or with fraction 5 of human thymic factor preparation. Cells were then washed three times. Viability was verified to be identical (>94%) in all tubes. A 3-day incubation with Con A was carried out.

Other characteristics of T cells were analyzed and their relationship with surface markers was determined. Cells with the capacity to proliferate after stimulation with Con A, PHA, or allogeneic cells reside within the HTLA$^+$, ERFC$^+$ population of peripheral blood, as shown by the virtually complete abrogation of these responses when HTLA$^+$ cells are killed or, alternatively, when ERFC have been eliminated (Table 2).

Human thymocytes are HTLA$^+$ and ERFC$^+$ but are able to respond to Con A only in low degree. This response could be significantly amplified by a 18-hr preincubation with the thymic factor preparation followed by its removal (Table 3). Comparable, but less dramatic, effects were observed with bone marrow cells from layers II and III of the bovine serum albumin gradient (Table 4). Similarly, thymic factors increased the response of both marrow cells and thymocytes to allogeneic stimulation in most experiments, even when the spontaneous response was extremely low. A slight increase of the PHA re-

Table 4. Induction and amplification of proliferative responses to stimulants of human bone marrow cells or thymocytes by treatment with thymic factors; effects of prior elimination of HTLA$^+$ cells or ERFC

Cells	Pretreatment	Response to: Con A	PHA	Allogeneic cells
BM cells (layers II and III)	Medium	+	+	±
	Human thymus fraction 5	++	+/++	+
	Anti-HTLA + C	0	0	0
	Removal of ERFC	0	0	0
Thymocytes	Medium	+	±	+
	Human thymus fraction 5	+++	+	++
	Anti-HTLA + C	0	0	0
	Removal of ERFC	0	0	0

Table 5. Separation of mitogen-responsive peripheral blood lymphocytes from those stimulated by allogeneic cells

Culture from day 0 to day 3	Culture from day 3 to day 6 or 8	Proliferative response, cpm × 10^{-3}
Medium	Medium	1
	PHA	172
	Con A	164
	Allogeneic cells	15
PHA	Medium	39
	PHA	41
	Con A	50
	Allogeneic cells	102
Con A	Medium	31
	Con A	35
	PHA	56
	Allogeneic cells	85

Human peripheral blood lymphocytes were cultured for 3 days with control medium, PHA, or Con A. Proliferating cells were inactivated by the BrdUrd and light technique on day 3. Remaining cells were cultured for 3 or 5 additional days with control medium or a second stimulant: PHA, Con A, or mitomycin-treated allogeneic cells. The incorporation of [^{3}H]thymidine was determined on the last day of the second culture period. A good proliferative response to allogeneic stimulation after inactivation of mitogen-responsive cells indicates that different lymphocyte subsets respond to phytomitogens and to allogeneic stimuli.

sponse also was often noted. Although we cannot completely rule out the possible proliferation of Con A$^+$ cells over the 18-hr period of preincubation, we interpret these results as suggesting that some HTLA$^+$, ERFC$^+$, Con A$^-$, or mixed leukocyte reaction-negative (MLR$^-$) cells have been converted into HTLA$^+$, ERFC$^+$, Con A$^+$, or MLR$^+$ cells. A smaller number of cells might also be induced to acquire the PHA response. In additional experiments, pretreatment of both marrow cells and thymocytes with anti-HTLA serum and complement or removal of ERFC completely abrogated the subsequent mitogenic and allogeneic responses that developed after thymic factor induction.

Comparisons of responses of peripheral blood lymphocytes to Con A, PHA, or allogeneic cells were performed by using an ablative technique (7). Lymphocytes were cultured for 3 days with a mitogen and then treated with 10^{-3} M BrdUrd and light to inactivate the proliferative cells; the remaining cells were stimulated for a second 3-day period with a mitogen or for a 5-day period with mitomycin-treated allogeneic cells (Table 5). When the same mitogen was used during the two periods (e.g., PHA–BrdUrd–PHA or Con A–BrdUrd–Con A), no significant additional response was observed at day 6, indicating ablation of each of the mitogen responses. When Con A was added after inactivation of PHA-responsive cells, or vice versa, little or no further stimulation was induced, indicating that in normal adult peripheral blood the subpopulations of PHA$^+$ cells and Con A$^+$ cells are approximately the same. In contrast, after inactivation of PHA$^+$ or Con A$^+$ cells, allogeneic stimulation led to a higher response, indicating that the cell populations responding to the two stimuli are separated. This effect was not due to differences in duration of the second culture because no better stimulation by PHA or Con A was noticed when these cultures were also terminated after 5 days of the second period (data not shown). The response to allogeneic stimulation not only was maintained after inactivation of phytomitogen-sensitive cells but was found to be even greater than the sum of the response left after mitogen-BrdUrd treatment and of the control

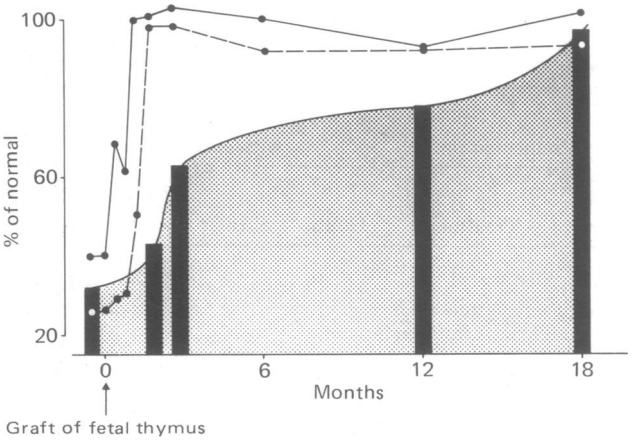

FIG. 1. Development of T cells with HTLA$^+$ phenotype (· — ·), capacity to form E rosettes (· - ·), and Con A responsiveness (black bars and stippled area) after fetal thymus transplantation in a patient with DiGeorge's syndrome.

allogeneic response. This enhanced effect was observed although the absolute concentration in allogeneic-responsive cells was kept identical in all tubes.

Insight into the development of T cells *in vivo* has been provided by immunological reconstitution of patients with severe immunodeficiency. Fig. 1 depicts T cell development in one such patient treated by fetal thymus transplantation for DiGeorge's syndrome. The infant, who had a profound T cell deficiency but normal T cell precursors (9), progessively acquired a normal number of mature T cells in the peripheral blood. These T cells derived from the recipient's precursor cells differentiating under the influence of the grafted thymus. The same sequence of differentiation events observed *in vitro* also occurred *in vivo*. T cells with an HTLA$^+$ phenotype appeared and at first did not form E rosettes. Later, the capacity to form E rosettes and still later the capacity to respond to Con A appeared. Nearly identical results were obtained after transplantation of compatible bone marrow in three patients with severe combined immunodeficiency (data not shown). In the latter cases, however, T cells derived from the donor's precursor cells were presumed to be differentiating *in vivo* under the influence of the recipient's thymus. The progession of the reconstitution also appeared to be slower than in the first case: development of HTLA$^+$ lymphocytes and ERFC required several weeks, and development of cells capable of responding to phytomitogens was much delayed. In the peripheral blood of the four patients studied, the allogeneic response developed either concurrently with the capacity to respond to Con A or slightly before it. The capacity to respond to PHA usually appeared soon afterward.

DISCUSSION

The present results, based on *in vitro* experiments and an analysis of patients after immunological reconstitution, confirm and extend prior analyses of both a sequential and a divergent differentiation of T cell subsets in man (11). Each stage of differentiation is envisioned to be dependent upon inducers, principally of thymic origin, and upon other regulatory factors.

The sequence of T cell characteristics described in Fig. 2 may be valid only for the methods used. For example, different methods for rosette formation (e.g., with neuraminidase-treated cells or with various conditions of incubation) may not characterize the same stage of differentiation. In addition, the

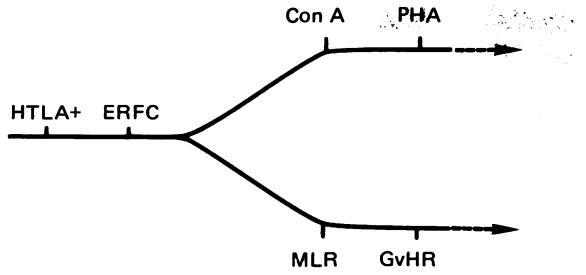

FIG. 2. Schematic of sequential and bifurcational development of human T cells. GvHR = graft-vs-host reaction.

location of the MLR in this scheme corresponds to the stage at which a maximal response is observed, and it seems likely that some degree of proliferation may also be induced by allogeneic stimulation in less-mature cells (12).

HTLA and E-rosetting capacity appear, like Thy-1 in the mouse, to be early characteristics of T cells that persist throughout most of the life-span of the cell. The $HTLA^+$ phenotype is acquired readily by T cell precursors, the destiny of which is already determined. The concentration of these differentiation antigens on the cell surface, as evaluated by absorption analysis (8), is high immediately after thymic influence and then decreases progressively as T cells mature, whereas the concentration of histocompatibility HLA antigens increases. As indicated by their sequence of development *in vitro* and *in vivo*, the appearance of HTLA precedes E-rosetting capacity.

Experiments using ablation of cells by anti-HTLA serum and removal of ERFC demonstrate that only after these characteristics are acquired does the lymphocyte develop the functional capacity to proliferate in response to stimulation by the mitogens PHA and Con A or by allogeneic cells. The responses to mitogens and to allogeneic stimulation, which appear nearly concurrently with differentiation *in vitro* and with development of the lymphoid system *in vivo* but which can be dissociated by the BrdUrd ablation technique, are properties of separate T cell subpopulations. To account for the development of those two subsets, we envision a bifurcational step after the acquisition of the E-rosetting capacity. Alternatively, an even earlier separation may exist, the HTLA marker and the E-rosetting capacity being acquired by the various T lymphocyte subsets.

Based upon the pattern of development observed in the reported experiments, we ascribe the following phenotypes to T-cell development:

T0: $HTLA^-$, E-rosette$^-$, MLR^-/Con A^-
T1: $HTLA^+$, E-rosette$^-$, MLR^-/Con A^-
T2: $HTLA^+$, E-rosette$^+$, MLR^-/Con A^-
T3m: $HTLA^+$, E-rosette$^+$, MLR^+/Con A^-
T3c: $HTLA^+$, E-rosette$^+$, MLR^-/Con A^+.

The temporal sequence of the appearance of these phenotypes is difficult to delineate precisely. From the *in vitro* results, each differentiation step may require only a few hours, but the *in vivo* data indicate that, as the lymphoid system is developed, several weeks may be needed for the full development of a large population of mature T cells of each subpopulation from precursor cells. In the thymus, the $HTLA^+$ phenotype and the E-rosetting capacity are acquired by virtually all lymphocytes. Although proliferative responses of small magnitude are also demonstrable in thymocytes, the immediate thymic cell is still relatively immature. Differentiation into a fully immunocompetent T cell seems to require further influence of thymic hormones, and possibly of other factors, in the periphery.

Table 6. Separation of PHA-responsive cells from GvHR-inducing cells in the mouse

Medium	Treatment on day 3	Injection into irradiated CBA mice (no. of mice)	Spleen index
Control	0	+(25)	1.75
	BrdUrd + light	+(22)	1.76
PHA	0	+(24)	1.83
	BrdUrd + light	+(35)	2.57

Spleen cells from C57 B1/6 mice were cultured for 3 days with PHA or control medium. Some cultures were treated with BrdUrd and light to inactivate proliferating cells. The cells were then injected intraperitoneally into lethally irradiated CBA mice. GvHR was evaluated by the measure of spleen indices with a method derived from that of Simonsen (17). In brief, the ratio of spleen weight to body weight was determined in each animal group, and it defined the relative spleen weight. The spleen index was then obtained by dividing the relative spleen weight of test animals from each group by the mean relative spleen weight of nine control irradiated mice injected with isogeneic cells. With this assay, the minimal spleen index to demonstrate a GvHR was 1.30. The spleen index obtained in the fourth group of mice was increased at a significantly higher degree than in the other groups ($P < 0.001$).

The demonstration of separate T-cell subpopulations responsible for phytomitogen responses and for allogeneic responses confirms suggestions from animal experiments using dissociation of T cells into subsets by differential density (13) or sensitivity to anti-Thy-1 alloantiserum (14). It is also in agreement with findings in normal fetal organs of various types and ages (15) and in peripheral blood of patients with immunodeficiencies (16).

Although full expression of the graft-vs-host reaction (GvHR) involved a cooperation of several cell subsets, it was thought of interest to try to define more precisely the place of the GvHR-inducing T cell in the above-mentioned scheme of differentiation. In the absence of definitive evidence in man, experiments were conducted in the mouse. Spleen cells were stimulated with PHA, treated with BrdUrd and light, and injected into lethally irradiated allogeneic recipients; then the splenic indices were determined (17). Inactivation of PHA-responsive cells did not result in a decrease of the GvHR (Table 6), indicating that GvH^+ cells are different from PHA^+ cells. The reason for the consistently increased MLR and GvHR after inactivation of PHA-responsive cells is not known. An explanation (7) postulates the presence of cells with a spontaneous suppressor activity for allogeneic responses among mitogen-sensitive cells. In unpublished animal experiments involving treatment of allogenically stimulated cells with BrdUrd and light, we have observed a more pronounced decrease of the capacity to induce a GvHR in the specific allogeneic recipient than in a third party. It is therefore likely that the GvH^+ characteristic, completely separated from the PHA^+ characteristic, is closely associated with the *in vitro* MLR response (Fig. 2). Occasionally, these two responses appear to be dissociated—e.g., when a GvHR develops in the absence of any apparent stimulation in mixed lymphocyte culture, after transplantation of cells from an HLA A, B, and D identical sibling into an immunodeficient patient. It should be noted that the MLR explores incompatibility only, or largely, at the D locus, whereas the GvHR can probably be induced by incompatibilities outside the major histocompatibility complex.

Furthermore, the GvHR assay appears to be more sensitive than the MLR. Nonetheless, only those T cells mature enough for an *in vitro* proliferative response to allogeneic stimulation appear to be capable of inducing a GvHR.

The characteristics tentatively located in this scheme of differentiation represent only a small proportion of the known characteristics of T cells. Based on this preliminary model, and using comparable ablative techniques, it may be possible to determine the place of other markers and other functions—e.g., proliferative response to nonallogeneic antigens, soluble mediator secretion, and helper, suppressor, and killer activities. From studies of Ly phenotypes of mouse T cells, it appears that different subsets are responsible for the helper and killer functions (18, 19). Furthermore, the suppressor T cell exhibits the same Ly phenotype as the killer T cell but, like thymus-independent lymphocytes (B cells), is Ia^+ (20). Further development of these studies in mice and man will probably demonstrate more than two subpopulations of T cells differentiating in parallel.

Whatever surface characteristic is used for the identification of a functionally distinct subpopulation of T cells, its expression may not be necessary for the associated function or for the process of differentiation itself. These characteristics may be merely markers and their low level of expression or absence may not necessarily reflect a block of T-cell differentiation. Such lack of the external expression of a marker or even of a function could explain some apparent exceptions to the described scheme, particularly in pathologic states. For instance, low E-rosette formation has been observed in the presence of normal MLR, Con A, and PHA responses.

This model of T-cell differentiation, mainly based on *in vitro* experiments, is consistent with results of investigations in patients after immunological reconstitution and with results of ontogenetic studies (12). The scheme must remain somewhat tentative in its design but it seems likely that it will be of help in the more precise characterization and classification of T-cell deficiencies, be they consequent to an early block in differentiation or to a later inhibition preventing the normal development of a T-cell subset.

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