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J Clin Invest. 1984;73(1):191-201. https://doi.org/10.1172/JCI111190.

Research Article

Unexplained, generalized lymphadenopathy in homosexual men, which can be a prodrome to the acquired immunodeficiency syndrome, is associated with impaired cell-mediated immunity, a low ratio of T helper-inducer to T suppressor-cytotoxic cells (defined by the T4 and T8 monoclonal antibodies), and hypergammaglobulinemia. We performed double-marker studies on T cells by using a panel of monoclonal antibodies (Ia, T17, TQ1, and Leu-8), which reportedly detect activation or functional subsets of the T4 and T8 T cell populations. The T4:TQ1- or T4:Leu-8- subset, which is the major helper subset for B cell responses, is normally represented in lymphadenopathy patients. A depression in the reciprocal subset, T4:TQ1+ or T4:Leu-8+, accounts for the T4 T cell defect. Similarly, the TQ1 and Leu-8 markers delineate the abnormality of T8 T cells: the T8:TQ1- or T8:Leu-8- subset is elevated, whereas the T8:TQ1+ or T8:Leu-8+ subset is normally represented. We found no evidence of excessive activation of T4 T cells by using the T17 or Ia monoclonal antibodies. We did find an overall increase in Ia-positive T cells; however, this was due to increased T8:Ia+ cells. In functional studies, immunoglobulin production induced by pokeweed was subnormal. Most lymphadenopathy patients had normal T helper cell function when combined with normal B cells. The dampened pokeweed responses could be partially explained by depression of the T4:TQ1+ (or T4:Leu-8+) subset […]

Immunoregulatory Subsets of the T Helper and T Suppressor Cell Populations in Homosexual Men with Chronic Unexplained Lymphadenopathy

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Abstract. Unexplained, generalized lymphadenopathy in homosexual men, which can be a prodrome to the acquired immunodeficiency syndrome, is associated with impaired cell-mediated immunity, a low ratio of T helper-inducer to T suppressor-cytotoxic cells (defined by the T4 and T8 monoclonal antibodies), and hypergammaglobulinemia. We performed double-marker studies on T cells by using a panel of monoclonal antibodies (Ia, T17, TQ1, and Leu-8), which reportedly detect activation or functional subsets of the T4 and T8 T cell populations.

The T4:TQ1$^-$ or T4:Leu-8$^-$ subset, which is the major helper subset for B cell responses, is normally represented in lymphadenopathy patients. A depression in the reciprocal subset, T4:TQ1$^+$ or T4:Leu-8$^+$, accounts for the T4 T cell defect. Similarly, the TQ1 and Leu-8 markers delineate the abnormality of T8 T cells: the T8:TQ1$^-$ or T8:Leu-8$^-$ subset is elevated, whereas the T8:TQ1$^+$ or T8:Leu-8$^+$ subset is normally represented. We found no evidence of excessive activation of T4 T cells by using the T17 or Ia monoclonal antibodies. We did find an overall increase in Ia-positive T cells; however, this was due to increased T8:Ia$^+$ cells.

In functional studies, immunoglobulin production induced by pokeweed was subnormal. Most lymphadenopathy patients had normal T helper cell function when combined with normal B cells. The dampened pokeweed responses could be partially explained by depression of the T4:TQ1$^+$ (or T4:Leu-8$^+$) subset (which has minor help-associated function) and/or greater than expected suppression. However, subnormal pokeweed responses could not be totally explained by immunoregulatory T cell abnormalities because we also found an intrinsic defect in the B cell responses of lymphadenopathy patients.

Received for publication 29 June 1983 and in revised form 31 August 1983.

J. Clin. Invest.

0021-9738/84/01/0191/11 $1.00
Volume 73, January 1984, 191–201

Introduction

The acquired immunodeficiency syndrome (AIDS)[1] is an acquired and profound defect in cellular immune function, with susceptibility to life-threatening, opportunistic infections and/or malignancy (1–10). AIDS was first recognized in the homosexual community and subsequently has involved intravenous drug abusers, Haitians, hemophiliacs, and other recipients of blood products (1–6, 9, 10).

A progressive lymphopenia associated with a loss of cell-mediated immune functions occurs. Particularly affected are those functions mediated by T lymphocytes, such as delayed-type hypersensitivity, proliferative responses to mitogens and recall antigens, alloreactivity, and cytotoxicity (1–3, 9–12). Alterations in the distribution of certain subpopulations of T cells, as defined by monoclonal antibodies, accompany the defect. The hallmark, or at least a most consistent finding, has been a depression in the ratio of T helper-inducer cells (defined by T4 or Leu-3 monoclonal antibodies) to T suppressor-cytotoxic cells (defined by T8 or Leu-2 monoclonal antibodies) (1–3, 7–12).

A paradox arises in attempting to correlate cell surface phenotype with either cell function or in vivo observations. One function of the helper-inducer population is to stimulate B cell proliferation and differentiation into immunoglobulin-producing cells (13–15). Yet, despite the profound depression in T4 or Leu-3 T cells, immunoglobulin levels in these patients are normal or elevated (1, 2, 7–10). One explanation is that the defect

1. *Abbreviations used in this paper:* AIDS, acquired immunodeficiency syndrome; C, complement; FACS, fluorescence-activated cell sorter; ILS, idiopathic lymphadenopathy syndrome; PHA, phytohemagglutinin; PWM, pokeweed mitogen.

involves a subset of T cells within the T helper-inducer population that is not directly involved in the helper function for B cells. Another possibility is that the remaining T cells somehow compensate for their numerical deficiency through excessive activation or proliferation.

Two monoclonal antibodies which detect the T cell antigens, TQ1 and Leu-8, define subpopulations of T helper-inducer cells. TQ1-negative or Leu-8-negative cells within the T helper-inducer population are primarily involved in the helper function for B cells (16, 17). Another marker, T17, is found on almost all peripheral T cells but is lost, after activation, by a subset of T cells involved in the helper function (18). Two additional markers, T9 and Ia, are not normally found on peripheral T cells but may become expressed with proliferation or after activation (18–22). We selected this panel of monoclonal antibodies to determine the distribution of markers within the T4 (helper-inducer) and T8 (suppressor-cytotoxic) T cell sets. These studies were performed on homosexual men presenting with generalized lymphadenopathy of unknown cause, the idiopathic lymphadenopathy syndrome (ILS). ILS can be a prodromal syndrome to AIDS (7, 12, 23, and Centers for Disease Control, AIDS Task Force, unpublished data), though it remains to be determined what percent actually go on to develop AIDS. We studied ILS rather than AIDS per se for three reasons: ILS patients have inverted T4/T8 ratios but are generally not lymphopenic and would yield enough lymphocytes from a reasonable amount of blood for the studies we envisioned; they do not have supervening opportunistic infections, which might complicate the interpretation of results; and, since this may be the earliest recognizable form of AIDS, we felt that any abnormalities found would more likely reflect proximate rather than secondary defects.

Methods

Patients. 13 patients with ILS were selected from a group of 50 ILS patients being monitored in Atlanta. The patients were homosexual or bisexual men with generalized lymphadenopathy of at least 3-mo duration, often with symptoms of fever, fatigue, and weight loss. Extensive evaluation, including lymph node biopsy, failed to reveal a known cause of the lymphadenopathy. None of the patients had any evidence of opportunistic infection at the time of study. In addition to a willingness to participate and be available for repeated testing if necessary, we used two criteria for selection: the patient must have an inverted T4/T8 ratio < 1.0 and must not be lymphopenic. These criteria were commonly found in the group of 50 ILS patients: T4/T8 ratios < 1.0 were found in 66% and lymphocyte counts > 1000 mm^{-3} were present in 94%. Controls studied in parallel with the ILS patients were healthy young adults in the same age range as the patients, but were otherwise not preselected.

Lymphocyte preparations for testing and statistical analysis. Mononuclear leukocytes were separated from peripheral blood by Ficoll-Hypaque sedimentation (24) and were either tested immediately or were cryopreserved until testing. All analyses involving the TQ1 and Leu-8 monoclonal antibodies were run on fresh cells because we had found that these markers can be lost on storage or culture. For the rest of the phenotype data, about half were obtained by using fresh cells and half were obtained by using cryopreserved cells. All functional assays were run on cryopreserved cells. Similarly processed normal control cells were included in all runs. Within each group (control and ILS), separate analysis of phenotype data from fresh or cryopreserved cells showed no differences.

T4 and T8 marker studies were done two times for all donors and three times for about half: once in the initial screening for selecting of ILS patients for the study, and once or twice more depending on whether the phenotyping was done entirely on fresh cells or partially on fresh cells and partially on cryopreserved cells. The between-run variation on fresh and cryopreserved cells was no greater than the between-run variation on cryopreserved cells tested on two occasions (3.6% for T4 and 2.9% for T8). Only one data point for each participant involving the T4 and T8 markers was used in statistical analysis, and the statistical results were equivalent regardless of which data set was used.

For each test, a minimum of 10 data points from the ILS and from the control group were used for statistical analysis. Three of the 13 ILS patients were not recalled for studies on fresh cells involving the TQ1 and Leu-8 markers: one developed *Pneumocystis carinii* pneumonia, one developed lymphoma, and one was taking steroids for atopic dermatitis at the time of recall. Computer-assisted statistical analysis of variance between groups used the Wilcoxon rank-sum test.

Monoclonal antibodies and fluorescence-activated cell sorter (FACS) analysis. The following murine monoclonal antibodies were used: α-T4, α-T8, α-T3, α-T6, (OKT4, OKT8, OKT3, OKT6) (Ortho Pharmaceutical Corp., Raritan, NJ); α-T11, α-Mo2, α-B1 (Coulter Corp., Hialeah, FL); α-Leu-8, α-Ia (α-HLA-DR, nonpolymorphic) (Becton-Dickinson & Co., Sunnyvale, CA); α-TQ1 (16), and α-T17 (18). All reagents were pretitered (regardless of manufacturer's suggested dilution) by using normal lymphocytes to determine optimal conditions for binding.

The binding of monoclonal antibodies to lymphocytes was analyzed by indirect immunofluorescence using a fluorescence-activated cell sorter (FACS-IV, Becton-Dickinson & Co.). Briefly, 5×10^5 peripheral blood mononuclear leukocytes were incubated in 25 μl-volume dilutions of monoclonal antibody (or combination of monoclonal antibodies) for 30 min at 4°C. Diluent buffer was 0.01 M PO_4, 0.15 M NaCl, pH 7.4 (PBS), containing 0.1% bovine serum albumin, 2% AB^+ serum, and 0.1% NaN_3. The cells were washed by centrifugation and resuspended in a saturating concentration of fluorescein-conjugated, human IgG-absorbed, goat α-mouse immunoglobulin reagent (25) for 30 min at 4°C. After being washed as above, the cells were analyzed in the FACS equipped with an argon laser emitting light at 488 nm and 200 mW. Fluorescence was detected with an S-20 response photomultiplier tube at 850 V with barrier filters at 520 and 530 nm. Fluorescence data was collected on 10,000 cells/test by "gating" on the forward low angle scatter peak containing viable lymphocytes. This gate maximizes lymphocyte detection and minimizes monocyte and dead cell inclusion. Because of the possibility that the distribution of lymphocytes might be different in ILS and controls by using forward angle scatter, we routinely monitored the gate containing larger cells and monocytes as well. We did not find any differences between ILS and controls in the number of T4- or T8-positive cells in the large cell population excluded by our gate.

The FACS data readout for each marker or marker combination is percentage of mononuclear leucocytes that are fluorescence-positive. Background staining with buffer or control murine ascites and the fluorescein-conjugate was, at most, 1.9% (average 0.7±0.5%). This was subtracted from all measurements. Although the FACS analysis is "gated" to eliminate (by light scatter) most monocytes from analysis, we included the Mo2 monoclonal antibody in all runs to monitor monocyte con-

Table IA. Determination of the Distribution of the TQ1 Marker within the T4 and T8 T Cell Sets: Monoclonal Antibody Combinations Used for Indirect Immunofluorescence and FACS Analysis

Tube No.	Monoclonal antibodies	Cell subsets detected				
		$T4^+:TQ1^+$	$T4^+:TQ1^-$	$T8^+:TQ1^+$	$T8^+:TQ1^-$	$T4^-:T8^-:TQ^+$
1	α-T4	+	+	−	−	−
2	α-T8	−	−	+	+	−
3	α-T4 + α-T8	+	+	+	+	−
4	α-T4 + α-TQ1	+	+	+	−	+
5	α-T8 + α-TQ1	+	−	+	+	+
6	α-T4 + α-T8+ α-TQ1	+	+	+	+	+
7	α-TQ1	+	−	+	−	+

Table IB. Determination of the Distribution of the TQ1 Marker within the T4 and T8 T Cell Sets: "Obligate Overlap" Method for Calculating the Subsets

Subset	Calculation (using FACS results of 1–7 above*)
$T4^+:TQ^+$	1 + 7 − 4 or 5 − 2 − (6 − 3)
$T4^+:TQ^-$	4 − 7 or 6 − 5
$T8^+:TQ^+$	2 + 7 − 5 or 4 − 1 − (6 − 3)
$T8^+:TQ^-$	5 − 7 or 6 − 4
$T4^-:T8^-:TQ^+$	6 − 3 or 6 − (1 + 2)

* Nos. 1–7 represent the FACS results obtained with monoclonal antibody combinations in tubes 1–7 after correction for background and monocyte contamination. Calculations yield percentage of lymphocytes (% Ly) and can be converted to percentage of T cells (% Ly × 100/% positive in tube 3) or absolute number (% Ly × absolute lymphocyte count/100).

tamination. The FACS data readouts for each marker were corrected to give the percentage of lymphocytes positive by multiplying all readouts by 100/100 − % Mo2-positive. This is a valid correction for any marker not found on Mo2-positive cells. We examined the reactivity of monocytes with our reagents by using the 90° light scatter and "gating" on the monocyte population. The T4 reagent reacted with a low level of fluorescence. Our cut-off for discriminating positive from negative was set above this level, and thus, T4-positive monocytes are excluded from analysis. Similarly, none of the other reagents, except α-Ia, stained monocytes with sufficient intensity to be registered as positive. Since Ia can be found on monocytes, the monocyte correction factor used would result in an overestimate of Ia-positive lymphocytes. However, because of the method of calculating the marker distribution within the T cell subsets (see below), this overestimate would be relegated to the non-T cell lymphocyte population. We used the monocyte correction because it permits the calculation of absolute cell counts for each marker from the peripheral leukocyte count and differential.

Distribution of Ia, T17, Leu-8, and TQ1 markers within the T4 and T8 T cell subsets. The monoclonal antibody combinations for FACS analysis and the calculations used are illustrated in Table I for the TQ1 marker (the Ia, T17, and Leu-8 FACS analyses and calculations are identical). The "obligate overlap" method of calculation is explained as follows: if one antibody detects x percentage of cells, if a second antibody detects y percentage of cells, and if both antibodies combined detect z percentage of cells, x plus y will equal z if the markers detected are found on separate cell populations. If $z < x + y$, there must be overlap, and $x + y - z$ percentage of cells must express both markers.

This method is subject to all the experimental constraints imposed in single marker studies (specificity and optimal titration of reagents, background staining, FACS variation, and accuracy). Two additional constraints pertain. One is that the use of combined antibodies must accurately detect the sum of cells positive for one, the other, and both markers. Steric hindrance between two antibodies binding to separate antigens on the same cell can occur and is not an uncommon problem when examining single cells for dual markers. The obligate overlap calculation remains valid, however, even if there is interference between the two antibodies, as long as the cell remains fluorescence-positive by virtue of one of the antibodies. Only if binding of two different antibodies somehow renders the cell antibody-negative are the calculations invalid. This seems remote, but it is formally possible that some mechanism such as excessive cocapping and shedding might render double-marked cells negative. This does not occur under the conditions we used, or at least we have ruled out interference of α-T4 and α-T8 binding by the Ia, T17, Leu-8, or TQ1 reagents. T4 or T8 reagents which were directly conjugated with fluorescein gave the same percent positivity whether incubated with or without unlabeled Ia, T17, Leu-8, or TQ1 reagents and unlabeled α-mouse immunoglobulin. The second constraint is that it is theoretically possible that cells giving a low level of fluorescence when stained with individual reagents might cross the cut-off threshold and be registered as positive when doubly stained. With all our reagents, only a small proportion of the cells hovered around the cut-off point. Because of the problem with T4-positive monocytes alluded to above, we set our cut-off well above the peak of fluorescence-negative cells. Compared with individually stained preparations, there were no shifts of the fluorescence-negative peak in doubly stained preparations such that a portion or "shoulder" of the peak crossed the cut-off; nor was there any measurable increment in the number of fluorescence-positive cells just above the cut-off. This is not to say that the phenomenon does not occur, but if it does, it cannot involve >2% of the cells.

The first calculation formula for each subset in Table I uses data obtained by using the two pertinent marker antibodies. The alternative calculation formulae which follow use data generated by using three markers (T4, T8, and either Ia, T17, Leu-8, or TQ1) and served as a validity check on the first calculation. To be valid, formulae involving

Table II. ILS: Lymphocyte Counts, T4/T8 Ratios, and Immunoglobulin Levels

			Immunoglobulins		
	Lymphocyte count	T4/T8 ratio	IgG	IgA	IgM
	mm⁻³			*mg/100 ml*	
ILS* group	1,746 (1,018–2,754)	0.60 (0.26–0.97)	2,122 (1,167–4,498)	340 (128–866)	230 (77–737)
Control* group	1,799 (1,035–2,500)	2.07 (0.98–3.21)	1,052 (660–1,290)	240 (105–396)	177 (103–280)
Normal values‡	1,903 (1,071–2,688)	2.17 (1.10–3.38)	1,047 (564–1,765)	177 (85–385)	126 (45–250)

* Results for the ILS patients and controls studied in parallel are given as means and ranges.
‡ Normal values for our laboratory are given as means and 95% confidence intervals.

three markers are based on the assumption that the T4 and T8 T-cell populations are mutually exclusive; i.e., combined staining with α-T4 and α-T8 equals the sum of staining with each reagent separately. In these experiments, these measurements differed from each other by an average of 2.2%. Data reported here use the first set of calculations, but the results and statistical analyses are essentially the same with all the modes of calculation.

In vitro assays of lymphocyte function. The pokeweed mitogen (PWM) culture system for the induction of immunoglobulin production as described by Rodriguez et al. (26) was used with minor modifications. Triplicate cultures containing 2×10^5 lymphocytes in 0.2 ml were cultured with PWM (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) in microculture trays for 7 d. For each lymphocyte donor, three doses of PWM (1:1000, 1:200, and 1:50 final dilutions of PWM) as well as cultures without PWM were tested. After 7 d, supernate medium was harvested and stored at −70°C until assayed for immunoglobulin by an enzyme-linked immunoadsorbant assay described by Kearney et al. (27).

In co-culture experiments, cultures contained 1×10^5 cells from each of two donors. In admixture experiments, T + B cells were separated by the *s*-2-aminoethylisothiouronium bromide hydrobromide-treated sheep erythrocyte rosetting method of Falhoff et al. (28). Separated T and B cells were then recombined for culture with PWM in both syngeneic and allogeneic combinations (2×10^5 T cells plus 0.5×10^5 B cells) as indicated in the text. In some experiments, $T8^+$ T cells were eliminated from the T cell population by treatment with monoclonal anti-T8 antibodies and rabbit complement under predetermined conditions for optimal immune cytolysis (29). The T and B cell separation procedures were monitored both phenotypically by FACS analysis as above and functionally by the inability of the separated cell populations when cultured alone to mount an immunoglobulin response.

Lymphocyte proliferative responses to the mitogens, phytohemagglutinin (PHA), conconavalin A, and PWM, and the antigens, *Candida*, tetanus, streptolysin O, herpes simplex virus, and cytomegalovirus, were assayed in microculture as detailed elsewhere (10).

Results

The initial laboratory observations for patients with ILS who were alluded to in the introduction are presented in Table II. The ILS patients that we studied have normal lymphocyte counts, depressed T helper-inducer/T suppressor-cytotoxic cell ratios, and normal (or supernormal) immunoglobulin levels (Table II). Moreover, total T cell, monocyte, and B cell numbers are normal as assessed by monoclonal antibodies specific for these populations (T3, T11, Mo2, and B1) (Table III). The phenotype data obtained by using monoclonal antibodies in Tables III–V are expressed both in terms of percentage and absolute number of lymphocytes expressing the phenotype, and where relevant, are also expressed as the phenotypic distribution within the T cell set or within a given T cell subset. Thus, the abnormal T4/T8 ratio in ILS is due to both a relative and an absolute decrease in $T4^+$ T cells and a relative and an absolute increase in $T8^+$ T cells (Table III, Fig. 1). We found no abnormality or increase in cells bearing the T6 antigen (immature cells or thymocytes) (30) or the T9 antigen (proliferating, transferrin receptor-positive cells) (20) (Table III). The overall distribution of the Ia, T17, TQ1, and Leu-8 phenotypes shown in Table III will be discussed in the context of their distribution within the T4 and T8 T cell sets.

T4 T cell subsets. Table IV presents data on the distribution of the Ia, T17, TQ1, and Leu-8 phenotypes or subsets within the T4 T cell set. Portions of this data are presented graphically in Fig. 1. The relative and absolute decrease in the T4 T cell population is due entirely to decreases in the $T4{:}TQ1^+$ or $T4{:}Leu\text{-}8^+$ subpopulation. There is no abnormality in the number of $T4{:}TQ1^-$ or $T4{:}Leu\text{-}8^-$ cells in ILS whether expressed as percentage of lymphocytes, percentage of T cells, or absolute numbers. Most T cells, including T4 T cells, are Ia^- and $T17^+$. We found no evidence that ILS patients, as a group, have increased numbers of Ia^+ or $T17^-$ T4 T cells. However, the two patients with the highest number of Ia^+ T4 cells also have the highest level of $T17^-$ T4 cells. These patients were otherwise similar to the ILS group in that they also have depressed numbers of $TQ1^+$ and $Leu\text{-}8^+$ T4 T cells.

We confined this study to those ILS patients with an inverted T4/T8 ratio, which is characteristic of the majority (66%) of the ILS patients we have seen. We have studied four additional patients with normal ratios and six ILS patients with normal levels of T4 T cells (the latter group includes two patients with inverted ratios). Inclusion of these patients into the ILS panel may be more representative of the ILS population as a whole, but is not germane to the rather narrow question we sought to answer: when T4/T8 ratios are inverted in ILS, what T4 and T8 T cell subsets are involved. Subset results in ILS patients

Table III. Single Marker Studies with Monoclonal Antibodies in ILS

Cell set	Putative specificity of monoclonal antibody	Percentage of lymphocytes			Percentage of T cells			Absolute No.		
		ILS	CON	*P*	ILS	CON	*P*	ILS	CON	*P*
T3	Pan T	71 (7)*	75 (6)	NS‡	100 (3)	100 (3)	NS	1,237 (359)	1,343 (358)	NS
T11	Pan T (E rosette receptors)	78 (9)	79 (7)	NS	100 (1)	99 (1)	NS	1,371 (351)	1,421 (403)	NS
T6	Immature T (thymocytes)	<1 (1)	<1 (1)	NS	<1 (1)	<1 (1)	NS	7 (11)	4 (6)	NS
T9	Proliferating cells (Transferrin receptor)	1 (1)	1 (1)	NS	2 (2)	1 (1)	NS	19 (17)	9 (10)	NS
Ia	B cells, monocytes, and activated T cells	33 (10)	21 (7)	0.0034	18 (13)	5 (6)	0.0279	587 (229)	389 (189)	0.0456
T4	T inducer cell set	24 (8)	48 (6)	0.0001	34 (11)	64 (9)	0.0001	412 (186)	869 (290)	0.0005
T8	T suppressor/cytotoxic cell set	46 (8)	26 (7)	0.0001	65 (10)	34 (9)	0.0001	804 (277)	455 (162)	0.0011
T17	Pan T (T cell subset)	78 (8)	79 (9)	NS	93 (3)	95 (6)	NS	1,341 (356)	1,411 (397)	NS
TQ1	T cell subsets	31 (9)	59 (10)	0.0006	35 (11)	61 (14)	0.0019	662 (162)	936 (411)	NS
Leu-8	T cell subsets	46 (11)	73 (12)	0.0007	49 (14)	73 (10)	0.0012	995 (261)	1,129 (396)	NS
B1	B cell	8 (5)	8 (4)	NS				139 (121)	150 (89)	NS
Mo2	Monocyte/macrophage							151 (81)	129 (68)	NS

Con, control.
* Mean (SD).
‡ NS, not significant.

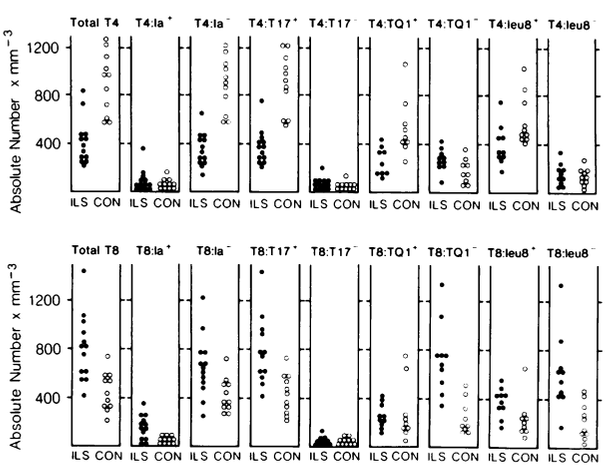

Figure 1. Subsets of the T4 and T8 T cell sets. The distribution of T4 T cell (*upper panel*) and T8 T cell (*lower panel*) subsets defined by the Ia, T17, TQ1, and Leu-8 monoclonal antibodies are shown for ILS patients (closed circles) and controls (*CON*) (open circles). Mean values, SD, and statistical comparisons for these data are in Tables IV and V.

with normal ratios tended to be intermediate between ILS (with inverted ratios) and controls, even if we compared them only with paired controls most closely matched for T4/T8 ratio or absolute T4 T cell count. Statistical analysis of the entire ILS data set had only minor effect on the means or statistical analysis presented in the tables. No significant difference became insignificant ($P > 0.05$) or vice versa.

T8 T cell subsets. The relative and absolute increase in T8 T cells in ILS, like the abnormality in the T4 T cell set, could be ascribed to particular subsets of T8 T cells defined by TQ1 and Leu-8 phenotypes. T8:TQ1$^-$ (or T8:Leu-8$^-$) cells are increased, whereas the number of T8 cells with the reciprocal phenotype, T8:TQ1$^+$ (or T8:Leu-8$^+$) is normal (Table V, Fig. 1). The majority of T8 T cells bear the T17$^+$ phenotype, and this population (T8:T17$^+$) was increased in ILS. T8:T17$^-$ T cells, representing a small proportion of T8 T cells, were similarly represented in ILS and controls. Both T8:Ia$^-$ T cells and T8:Ia$^+$ T cells were significantly elevated in ILS (Table V, Fig. 1). Of note is the fact that the overall increase in Ia-positive lymphocytes in ILS (Table III), a marker not ordinarily found on T cells, is due solely to an increase in Ia$^+$ T cells, and this increase can be attributed almost entirely to an increase in the T8:Ia$^+$ T cell population.

Functional studies. Lymphocyte proliferative responses to the mitogens, PHA, conconavalin A, and PWM, and responses

Table IV. $T4^+$ T Cell Subsets in ILS

	Percentage of lymphocytes			Percentage of T cells			Percentage of $T4^+$ cells			Absolute No.		
T cell subset	ILS	Con	*P*	ILS	CON	*P*	ILS	CON	*P*	ILS	CON	*P*
$T4^+$	24 (8)*	48 (7)	0.0001	34 (11)	64 (9)	0.0001	100	100		412 (186)	869 (290)	0.0005
$T4^+$:Ia^+	3 (8)	1 (3)	NS‡	4 (11)	1 (4)	NS	11 (26)	1 (6)	NS	64 (140)	15 (64)	NS
$T4^+$:Ia^-	21 (7)	47 (6)	0.0001	29 (11)	63 (8)	0.0001	89 (26)	99 (6)	NS	348 (157)	855 (280)	0.0001
$T4^+$:$T17^+$	21 (7)	46 (7)	0.0001	30 (9)	62 (9)	0.0001	91 (7)	96 (4)	NS	366 (147)	835 (277)	0.0001
$T4^+$:$T17^-$	3 (3)	2 (2)	NS	3 (4)	2 (3)	NS	9 (7)	4 (4)	NS	47 (56)	35 (44)	NS
$T4^+$:$TQ1^+$	14 (6)	33 (7)	0.0008	19 (9)	42 (11)	0.0011	52 (22)	72 (18)	0.0222	289 (135)	530 (259)	0.0220
$T4^+$:$TQ1^-$	12 (6)	13 (9)	NS	17 (7)	16 (11)	NS	48 (22)	28 (18)	0.0222	262 (108)	183 (101)	NS
$T4^+$:Leu-8^+	19 (7)	39 (7)	0.0003	27 (10)	49 (9)	0.0005	72 (18)	82 (9)	NS	403 (168)	604 (208)	0.0156
$T4^+$:Leu-8^-	7 (4)	8 (4)	NS	10 (6)	11 (5)	NS	28 (18)	18 (9)	NS	148 (92)	131 (73)	NS

CON, control.
* Mean (SD).
‡ NS, not significant.

to a panel of five recall antigens were depressed in ILS patients (Table VI). Mitogen-induced responses were, on average, less than half that of simultaneously tested controls. Most normal donors respond to at least one (98%) or two (87%) antigens in the panel of five which we tested. This panel included cytomegalovirus and herpes simplex virus antigens, for which there was serologic evidence of exposure in all the ILS patients. Half of the ILS patients did not respond to any of the five antigens.

The PWM-induced production of immunoglobulin by ILS lymphocytes, as compared with control lymphocytes, is shown in Fig. 2. Both the observation of normal immunoglobulin levels in ILS (Table II) and the demonstration of normal numbers of T4 T cells with the helper phenotype (Table IV) would support the prediction that PWM-induced immunoglobulin production in ILS patients would be normal. Clearly, it was not (Fig. 2). The next series of experiments were designed to determine whether the poor in vitro immunoglobulin responses were due to excessive suppressor cell activity, T helper cell incompetence, B cell incompetence, or some combination of all three.

Two types of experiments were done to measure suppression or suppressor effects in ILS. In co-culture experiments, lymphocytes from four ILS patients and four controls were each cultured with an equal number of cells from each of four normal donors. Peak PWM-induced immunoglobulin levels in the co-cultures were compared with the responses expected from an average of the two donors cultured alone. 16 data points were generated for each group. Control cultures generated 133±34% of the expected response, whereas ILS patients' cells, when co-cultured with normal cells, generated 74±43% of the expected response ($P < 0.01$). Depletion of monocytes by plastic adherence before co-culture with normal cells did not change the results.

In the second type of experiment, we eliminated T cells with the suppressor phenotype ($T8^+$ T cells) to determine whether this would restore PWM responses to normal. We separated T

Table V. $T8^+$ T Cell Subsets in ILS

	Percentage of lymphocytes			Percentage of T cells			Percentage of $T8^+$ cells			Absolute No.		
T cell subset	ILS	CON	*P*	ILS	CON	*P*	ILS	CON	*P*	ILS	CON	*P*
$T8^+$	46 (8)*	26 (7)	0.0001	65 (10)	34 (9)	0.0001	100	100		804 (277)	455 (162)	0.0011
$T8^+$:Ia^+	8 (5)	1 (3)	0.0178	11 (7)	2 (4)	0.0165	17 (14)	3 (16)	0.0138	139 (97)	29 (50)	0.0289
$T8^+$:Ia^-	38 (9)	24 (6)	0.0004	54 (12)	32 (7)	0.0005	83 (14)	97 (16)	0.0138	664 (246)	426 (134)	0.0099
$T8^+$:$T17^+$	45 (7)	24 (8)	0.0001	63 (10)	32 (9)	0.0001	96 (5)	95 (9)	NS‡	786 (286)	434 (169)	0.0017
$T8^+$:$T17^-$	2 (3)	1 (2)	NS	3 (4)	2 (2)	NS	4 (5)	5 (9)	NS	34 (43)	21 (32)	NS
$T8^+$:$TQ1^+$	12 (5)	15 (8)	NS	17 (6)	19 (10)	NS	27 (11)	48 (21)	0.0143	772 (387)	251 (138)	0.0008
$T8^+$:$TQ1^-$	34 (9)	16 (7)	0.0011	47 (12)	21 (9)	0.0004	73 (11)	52 (21)	0.0143	772 (387)	251 (138)	0.0008
$T8^+$:Leu-8^+	18 (6)	17 (8)	NS	25 (9)	22 (9)	NS	41 (16)	56 (22)	NS	381 (116)	283 (201)	NS
$T8^+$:Leu-8^-	28 (11)	14 (8)	0.0052	39 (14)	17 (10)	0.0022	59 (16)	44 (22)	NS	650 (420)	230 (154)	0.0025

CON, control.
* Mean (SD).
‡ NS, not significant.

Table VI. Lymphocyte Proliferative Responses

	Antigen-induced responses (five antigens tested)*: cumulative frequency of responders to						Mitogen-induced responses: mean (SD) of normalized responses‡		
	Five antigens	Four or more antigens	Three or more antigens	Two or more antigens	One or more antigens	No antigens	PHA	ConA	PWM
	%								
Lymphadenopathy group	10	20	50	50	50	50	83 (41)	94 (63)	43 (44)
Control group	40	40	80	100	100	0	167 (60)	224 (88)	87 (40)
Normal values	13	43	73	87	98	2	158 (53)	229 (216)	104 (45)

* Antigens: *Candida*, tetanus, streptolysin 0, herpes simplex virus, cytomegalovirus. A stimulation ratio (counts per minute in cultures with antigen/counts per minute in cultures without antigen) of >3.0 is a positive response.
‡ Mitogens: PHA, conconavalin A, and PWM. Responses are "normalized" by expressing them as the percentage of the response of lymphocytes from a single donor which was included in all runs.
Con A, concavalin A.

and B cells, treated the T cells with α-T8 monoclonal antibody plus complement (C), and recombined them with autologous B cells. Both control and ILS patients' PWM responses were increased by depletion of $T8^+$ T cells. This increase was proportionately greater in ILS than in controls: T8 T cell depletion resulted in a 120% increase in immunoglobulin levels in ILS and a 60% increase in controls. ILS responses, though increased, were not restored to control levels. Thus, while there does appear to be more than the expected amount of suppression in ILS, suppression cannot totally explain the poor in vitro immunoglobulin responses to PWM.

To assess T helper cell and B cell function in the PWM system, T or B cells from ILS patients were recombined with allogeneic B or T cells from normal donors. In the assay for T helper cell function, T cells from ILS patients are cultured with normal B cells. The B cells in this assay were from a single donor whose B cells responded fairly consistently in allogeneic combination with normal T cells and were preselected for this property. Similarly, B cell competence was assessed in allogeneic cultures with a normal donor's T cells, which were preselected for consistent helper activity. The normal T cells and B cells in both assays were not from the same donor, and despite the preselection, did not always restore the PWM-induced immunoglobulin response, even in combination with other normal donors (Table VII). This has been observed by others as well (31, 32). In the assay for T helper cell competence, T4 T cells from six of eight ILS patients and six of eight controls stimulated a normal donor's B cells in the PWM system. In the assay for B cell competence, only two of eight ILS patients' B cells responded to PWM in the presence of normal allogeneic T4 T

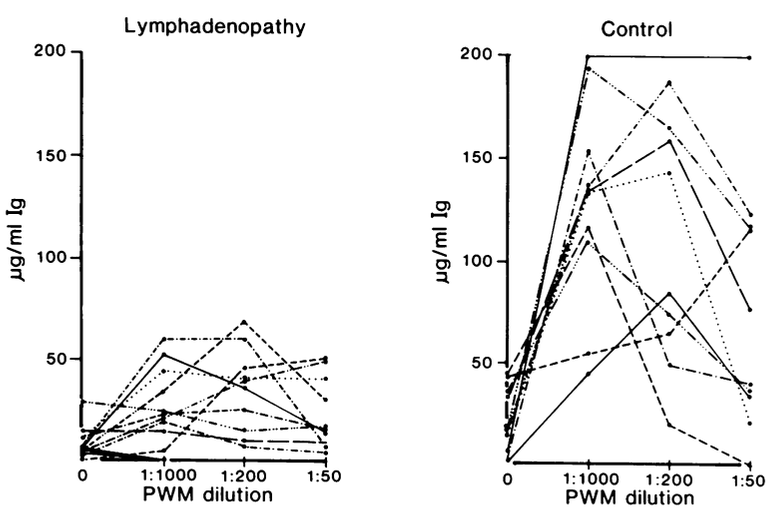

Figure 2. PWM-induced in vitro immunoglobulin response. Individual PWM-induced immunoglobulin responses in lymphocyte cultures from ILS patients (*left*) and normal controls (*right*) are shown. Statistical comparison between groups for each concentration of PWM are: unstimulated cultures, $P = 0.0347$; 1:1000 PWM, $P = 0.0002$; 1:200 PWM, $P = 0.0006$; 1:50 PWM, $P = 0.0175$.

Table VII. PWM-induced Immunoglobulin Responses in Cultures of Allogeneic T and B Cells

Culture combinations*		
Cells assayed	Cultured with	Immunoglobulin responses
		μg/ml
T4 T cells from eight ILS patients	B cells from a normal donor	144, 108, 67, 31, 13, 5, <1, <1
T4 T cells from eight controls	B cells from a normal donor	136, 112, 38, 25, 21, 20, <1, <1
B cells from eight ILS patients	T4 T cells from a normal donor	69, 5, <1, <1, <1, <1, <1, <1
B cells from eight controls	T4 T cells from a normal donor	52, 24, 20, 13, 10, 11, 2, <1

* T and B cells were separated and T cells were treated with α-T8 + C (T4 T cells) as described in the text. 2×10^5 T4 T cells and 0.5×10^5 B cells were combined as indicated. Peak pokeweed responses were assayed at 7 d. Results are a compilation of four experiments. Parallel experiments using whole T cells (rather than α-T8 + C-treated T cells) gave similar results.

cells, whereas seven of eight control B cells responded (Table VII). These experiments were also performed with whole separated T cells (rather than α-T8 + C-treated T cells) with the same response. In the separation procedure we used, monocytes sort with the B cell preparation. The poor responses of ILS patients' B cells in allogeneic cultures were not changed by partial depletion of monocytes from these preparations, nor was the response restored by supplementing monocyte-depleted cultures with normal monocytes (a 2,000 R irradiated B cell preparation syngeneic with the normal T cells in the assay). Finally, the poor PWM responses are not due to differences in the kinetics of the PWM response. However, ILS cells cultured in the absence of PWM do spontaneously secrete more immunoglobulin than controls. Four of five ILS cultures had detectable supernate immunoglobulin in the absence of PWM at 24 and 48 h (0.2–8.7 μg/ml), whereas one of five ILS cultures and five of five control cultures did not (<0.1 μg/ml). After 7 d in culture, however, spontaneous immunoglobulin secretion is less in ILS than controls (see responses in cultures without PWM in Fig. 2).

Discussion

The immune response is regulated by a series of interactions between phenotypically and functionally distinct subpopulations of T cells (13–15, 33–35). Two major subclasses of T cells regulate the production of immunoglobulin by B cells. Human T helper cell function is found exclusively in cells identified with the monoclonal antibody reagents, T4 or Leu-3 (14, 15). In contrast, the reciprocal T cell population (T8 or Leu-2) does not provide help, but contains cells which down-regulate or suppress the immunoglobulin response (34, 35). On the simple assumption that immunoglobulin levels observed in vivo are the net result of a balance between helper and suppressor regulation, one might predict that syndromes characterized by an inverted T4/T8 ratio would have subnormal immunoglobulin levels. Quite the opposite is the case (Table II). While perhaps unexpected, this should not be entirely surprising. For one thing, the functions of these T cell sets are defined as isolated cells in vitro, and it is not at all clear what the relative influence T4 and T8 T cell numbers, their relative proportions, their distribution, and other mechanisms unrelated to T cells have on in vivo immunoglobulin homeostasis. Secondly, T4 and T8 T cell populations are themselves functionally heterogeneous, and an overall perturbation of T4 or T8 T cell numbers may or may not affect certain subpopulations within these T cell sets.

In this study, we have addressed the latter issue. By using a select panel of monoclonal antibodies that define functional subsets within the T4 and T8 T cell sets, we find that the T4 T cell abnormality in ILS is due exclusively to a depression in a subset that, reportedly, is not primarily involved in helper function for B cells. Gatenby et al. (17) reported that the numerically minor Leu-8$^-$ T helper cell subset provides the majority of help for in vitro immunoglobulin production. Reinherz et al. (16) reported similar data for the T4:TQ1$^-$ subset. We find the T4:TQ1$^-$ and T4:Leu-8$^-$ subsets to be normally represented in ILS (Table IV, Fig. 1). Depression of the T4:TQ1$^+$ or T4:Leu-8$^+$ subset accounts entirely for the abnormality in T4 T cells in ILS. Though not the prominent helper subset, TQ1$^+$ or Leu-8$^+$ T4 T cells do contribute some helper effect for B cells (16, 17). This may partially explain why PWM responses were subnormal in ILS (Fig. 2). Other than having a minor helper effect and being the major T cell subset reactive in the autologous mixed lymphocyte reaction, the functions of the T4:TQ1$^+$ or T4:Leu-8$^+$ T cell subset have not been determined (16, 17). The recent report by Gupta and Safai (11) of defective autologous mixed lymphocyte reaction reactivity in AIDS patients with Kaposi's sarcoma is consistent with our phenotype data. It has been presumed by others that the T4:TQ1$^+$ or T4:Leu-8$^+$ subset subserves other T cell inducer functions in cell-mediated immunity including delayed-type hypersensitivity reactions and the induction of suppressor or cytotoxic T cells (16, 17).

Although the TQ1 or Leu-8 marker defines a functional subset of peripheral T4 T cells in freshly-drawn, normal blood, it is not known how stable these markers are on the T4 T cell

subset. It is possible that, after activation, these markers are lost and that the reduction in $TQ1^+$ or Leu-8^+ T4 T cells in ILS reflects a state of activation, rather than depletion of this subset. If so, we did not find concordance with several other markers of T cell activation. After activation, a portion of the T4 T helper cell population expresses the Ia antigen (21, 22), and a portion loses the pan T cell marker, T17 (18). T helper cell activity is not confined to these subpopulations. Both Ia^+ and Ia^- T4 subsets (and $T17^+$ and $T17^-$ T4 subsets) are required for optimal help for B cells (18, 21). Two ILS patients had relative increases in T4:Ia^+ cells. These same patients had the greatest numbers of T4:$T17^-$ T cells. However, we found no evidence that ILS patients, as a group, had increased numbers of Ia^+ or $T17^-$ T4 T cells (Table IV, Fig. 1), nor did they have increases in $T9^+$ cells, another marker of activated or proliferating cells (Table III) (20).

As mentioned, T4:Ia^+ and T4:$T17^+$ T cells participate with their respective reciprocal T4 subsets in the helper effect. In addition, they induce $T8^+$ T cells to exert feedback suppression of immunoglobulin synthesis in a model analogous to that described in the mouse (18, 22, 36, 37). One postulate that might reconcile the depressed T4 T cell numbers with the hypergammaglobulinemia found in ILS would be that the T4 T cell defect resides in the helper subpopulation, which also induces feedback suppression. Although we found no abnormality in Ia or T17 expression on peripheral T4 T cells, this does not militate against an abnormality in the feedback suppressor circuit. It is entirely possible that, after activation, Ia and T17 markers are abnormally expressed and that the feedback suppression pathway is defective in ILS. Such experiments are beyond our current capabilities. A functional assay for the feedback suppressor pathway would require, at best, an autologous system in which T8 suppressor cell activity is not already elevated and in which B cells are uniformly responsive. Neither requisite is met in ILS patients. It is not yet known whether the activated feedback suppressor inducers described with α-Ia and α-T17 reagents belong to the T4:$TQ1^+$ (or T4:Leu-8^+) subset, though it seems likely. (The TQ1 marker was named TQ1 by analogy to the Qa1 antigen, which defines a feedback suppressor inducer subset in the mouse [16, 36, 37]).

The relevance of abnormalities in T8 T cell phenotypic subsets is unclear because, for the most part, the functional role of these subsets has not been determined. Both T8:Ia^+ and T8:Ia^- T cells were elevated in ILS (Table V, Fig. 1). The elevation in T8:Ia^+ T cells accounts entirely for the overall increase in Ia^+ T cells in ILS (Tables III and V). Ia expression may reflect activation of T8 T cells which are functionally unrelated to B cell regulation (cytotoxic cells or suppressors of cell-mediated reactions) or activation of suppressors of B cell responses. We did find greater than the expected amount of suppression by ILS lymphocytes in co-culture experiments. In addition, elimination of $T8^+$ T cells resulted in a greater increase in PWM responses in ILS than in controls, though $T8^+$ T cell elimination did not fully restore PWM responses in ILS to normal. Gatenby et al. (17) demonstrated that both T8:Leu-8^+ and T8:Leu-8^- subsets are required for suppression of immunoglobulin responses. Otherwise, the functions of the TQ1 or Leu-8 T8 subsets have not been determined.

The allogeneic recombination experiments should be interpreted cautiously. In our hands, B or T cells from two unrelated normal donors, both of which give excellent in vitro immunoglobulin responses when combined in autologous cultures, do not uniformly respond when mixed in allogeneic cultures. This is reproducible for a given donor pairing, and some B and T cell donors function more consistently than others. The PWM system is not subject to allogeneic T cell-B cell restrictions (31, 32, 38), and judging from the magnitude of our positive responses, our culture conditions do not seem to be suboptimal. Whatever the explanation, we believe this has been the experience of most investigators using this methodology (31, 32, 38). Bearing this in mind, the most conservative interpretation of the allogeneic recombination experiments (Table VII) is that T cells of some ILS patients function normally as helper cells in the PWM system, and as a group, appear to be as consistent as control T cells in this assay. ILS patients' B cells, on the other hand, were consistently poor responders compared with control B cells, which responded most of the time. Our B cell preparations contain monocytes, and it is possible that the "B cell defect" represents a defect of monocyte function rather than of B cells per se. Neither partial depletion of monocytes from the B cell preparations nor supplementing these cultures with normal monocytes reversed the unresponsiveness of ILS patients' B cell preparations. There are no other published studies of allogeneic combination experiments in ILS; however, Mildvan et al. (8) did study a single AIDS patient. They found increased suppressor activity, decreased T helper function, and though it was not emphasized, depressed B cell responsiveness in a PWM system (8).

The interpretation of B cell incompetence presents a new irony. Why, if B cells are defective, are in vivo immunoglobulin levels normal or elevated? Bearing in mind that the interpretation of B cell incompetence is made in the context of how the B cells are assayed, the PWM system may not be relevant in all respects to what occurs in vivo. Though refractory to helper signals generated in the HLA-unrestricted, antigen-nonspecific PWM system, it is possible that ILS patients' B cells (or subpopulations of B cells) are otherwise activated or capable of activation. That is, they may be preactivated in vivo and refractory to additional triggering by PWM in vitro, or they may be responsive to other T-dependent pathways of activation or to direct T-independent polyclonal activators. We have found a greater rate of spontaneous immunoglobulin secretion in ILS than in controls. Thus, it seems quite possible that B cells may be already polyclonally activated in vivo (accounting for the hypergammaglobulinemia) and refractory to additional triggering by PWM. Infection with Epstein-Bar virus sets a precedence for this type of scenario: polyclonal activation of B cells, hypergammaglobulinemia, poor PWM responsiveness, and inverted T4/T8 ratios (38, 39). The only additional information on B cells that we have is that they are present in normal numbers in ILS (Table III) and that we found no differences between ILS and controls in TQ1 or Leu-8 markers on non-T lympho-

cytes. TQ1 and Leu-8 are found on a small proportion of B cells (16, 17). We have not determined the responsiveness of ILS to a variety of B cell stimulators. Such an analysis is beyond the scope originally envisioned for this study, but another group is actively pursuing this important question. Lane et al. (40), in a brief communication, report that B cells from AIDS patients have a high rate of spontaneous immunoglobulin production, but are otherwise poor responders in vitro to both PWM and the T-independent B cell activator, *Staphylococcus aureus* Cowan I.

Our original premise that the paradox of abnormally low T4/T8 ratios in the face of polyclonal hypergammaglobulinemia might be reconciled by analysis of helper subsets within the T4 T cell set appears to have been borne out by phenotypic analysis. The T4:TQ1$^-$ or T4:Leu-8$^-$ subset, which provides the majority of help for B cell responses, is normally represented, whereas the reciprocal population, T4:TQ1$^+$ or T4:Leu-8$^+$, which has only minor help-associated function, is depressed. Our functional assays, however, did not conform to expectations and suggest a much more complicated situation than phenotypic analysis would indicate. PWM-induced immunoglobulin responses were subnormal in ILS (Fig. 2). Some (if not all) ILS patients had normal helper function, and the dampened PWM responses might be partially explained by depression of the reciprocal help-associated T4:TQ1$^+$ or T4:Leu-8$^+$ subsets and/or greater than expected T8 T cell-mediated suppression. Were it not for the B cell defect, which cannot be completely explained by immunoregulatory T cell abnormalities, we might have suggested that the prime cellular defect in ILS relates to the reciprocal depression and elevation of the T4:TQ1$^+$ (or T4:Leu-8$^+$) and T8:TQ1$^-$ (or T8:Leu-8$^-$) subsets, respectively. While this is still possible, the striking B cell functional defect in vitro and the possibility that B cells are preactivated in vivo begs the question of what is the prime defect in ILS. A perturbation in one cell type may have a ripple effect throughout the immune network, and there is some precedence for an apparently primary B cell defect or infection to result in similar immunoregulatory T cell abnormalities—notably, congenital agammaglobulinemia (38, 41, 42) and Epstein-Barr virus infection (38, 39).

It is our bias that the primary insult in ILS and AIDS is identical and that the syndromes belong on a continuum or spectrum in time and/or severity of the same disease process. This study has focused on those ILS patients with a defined T cell abnormality (i.e., inverted T4/T8 ratios), in order to ask a rather narrow question: when T4/T8 ratios are inverted in ILS, what T cell subsets are involved. The relationship of the phenotypic abnormalities that we found to AIDS or to ILS associated with normal T4/T8 ratios has not been fully determined, largely for technical or logistical reasons. We have studied several AIDS patients with these same markers. Most of these patients have had profound depression of T4 T cells (T4/T8 ratios < 0.1) and lymphopenia, and thus, all T cell subsets are depressed. Three AIDS patients with T4/T8 ratios of 0.2–0.7 which we have studied have had the same T cell subset distribution as the ILS patients. Low, if not inverted, T4/T8 ratios are characteristic of ILS. The majority (66%) have inverted ratios, and the remainder have ratios at the low end of the normal range. Only 1 of 50 ILS patients that we have seen presented a ratio > 2.0. Of those with normal ratios, at least half have developed inverted ratios if followed for 3–6 mo. If the few ILS patients with normal ratios that we have studied are added to the ILS data set, the statistical validity of our observations is not changed. Thus, we have some reason to believe that the phenotypic abnormalities that we found are a general phenomenon in ILS. It remains to be determined if these abnormalities are unique to ILS, AIDS, or AIDS-related syndromes or if they are also found in other diseases associated with inverted T4/T8 ratios. The natural history of ILS is as yet unclear and prospective studies have been initiated by several groups. It is possible that detection of these subsets might be of greater value than T4/T8 ratios (or other tests) in predicting recovery or the onset of AIDS in ILS patients or in the discrimination of AIDS in high risk groups.

Acknowledgments

We thank Ms. Cecile Ivey for typing the manuscript; Mr. Robert Holman and Ms. Marjorie Hubbard for statistical analysis; Dr. Gideon Goldstein for his suggestions and generous supply of the T17 monoclonal antibody; and the Centers for Disease Control AIDS Activity Staff for their support and for reviewing the manuscript.

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