Development and characterisation of T lymphocyte lines from human small intestinal biopsies

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SUMMARY T lymphocyte lines were developed from human small intestinal biopsies obtained at the time of gastroduodenoscopy. Lines were established as outgrowths from biopsy specimens in microculture using a combination of T cell mitogens, indomethacin, interleukin 2 (IL-2) and autologous irradiated feeder cells. The predominant phenotype of T cells after six to 12 weeks in culture was CD2, CD3, and CD4 positive. Functionally, these T cell lines secreted IL-2 in response to stimulation with phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) in combination, but not to PHA or PMA alone. Two to 7% of cells in each line expressed natural killer (NK) cell-associated markers – that is, Leu 7, CD16, and Leu 19. Moreover, such cells were cytotoxic for NK sensitive targets, but not for NK resistant targets or allogeneic or autologous Epstein Barr Virus (EBV) transformed B cells. Sufficient numbers of small intestinal lymphoid cells for study were previously available only from patients undergoing surgical resections of the small bowel. The ability to isolate and culture human small intestinal T lymphocytes from gastroscopic intestinal biopsies provides an important new tool for studies of human small intestinal lymphocytes.

T lymphocytes in the human small intestine have been studied using immunohistochemical methods. CD4 bearing T lymphocytes – that is HLA class II restricted T lymphocytes, predominate in the lamina propria whereas CD8 bearing lymphocytes – that is, HLA class I restricted T lymphocytes, predominate in the intraepithelial region.^{1–4} The CD4 and CD8 markers correlate with the MHC restriction of T lymphocytes that usually, but not exclusively, mediate helper/inducer and cytotoxic/suppressor functions respectively.^{5,6}

Functional studies of human small intestinal lymphocytes have been limited as access to large numbers of such cells has been possible only in patients undergoing surgical resection. Further, experiments carried out on small intestinal lymphocytes freshly isolated from surgical specimens have produced conflicting results. For example, it has been controversial whether or not the human intestinal mucosa contains cells that mediate natural killer (NK) activity.^{7–12} Differences in cell separation pro-

cedures – that is, enzymatic or mechanical, and the proximity of resected specimens to disease may contribute to the variable results among studies.

We describe herein a system for the initiation and maintenance of human T lymphocyte lines from small intestinal biopsy specimens obtained at the time of gastroduodenoscopy. In addition, we report on the initial phenotypic and functional characteristics of these cell lines.

Methods

MONONUCLEAR CELLS

Peripheral blood mononuclear cells (PBMC) were separated from heparinised blood by centrifugation on Ficoll hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradients. Peripheral blood mononuclear cells were stored at –70°C in fetal calf serum (FCS) containing 5% dimethyl sulphoxide, until used as feeder cells.

T CELL LINES

Two to four biopsies, 3–5 mm in diameter, were obtained from the distal duodenum of each subject at the time of flexible gastroduodenoscopy. Two sub-

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jects (BH, ROPE) had coeliac disease, one had oesophageal carcinoma (ALS), one had antral gastritis (AH), one had a gastric ulcer (EJ), and one was endoscopically and histologically normal (PR). Both coeliac patients had partial villous atrophy on histology. Neither patient was adhering to a strict gluten free diet. Biopsies were placed in complete medium – that is, RPMI 1640 containing penicillin 100 U/ml, streptomycin 100 µg/ml kanamycin 100 µg/ml, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine and Hepes buffer 1 mg/ml, supplemented with 10% FCS and then divided into small fragments of approximately 2 mm diameter. Each fragment was placed in an individual well of a 96-well U-bottomed microwell plate (Costar) containing 200 µl complete medium supplemented with 10% pooled human serum. Ten micrograms per millilitre PHA (Sigma Chemical Co, St Louis, Mo), 1 µg/ml concanavalin A (Con A) (Calbiochem, La Jolla, CA), and 1 µg/ml indomethacin (Sigma) was added to each well. After three days, 10^5 autologous PBMC, irradiated with 4000R, were added to each well as feeder cells. On day 5 of culture, medium was supplemented with 20% MLA-144 (American Type Tissue Culture Collection (ATCC), Rockville, MD) supernatant as a source of IL-2, and cells were cultured for an additional five days. On day 10 of culture, biopsy fragments were removed and lymphocytes remaining in the fragments were obtained by agitating through a 21 gauge needle. T cells grew out from 50–60% of the tissue fragments. Cells were then pooled and cultured for 10 day cycles with irradiated PBMC as feeder cells. PHA, Con A, and indomethacin at the above described doses were added on day 1, and IL-2 was added on day 5 of the cycle. Lymphocyte viability within individual biopsy fragments was assessed by fluorescence microscopy after staining fragments removed from the microtitre wells with ethidium bromide 10 µg/ml and acridine orange 3 µg/ml. Using this protocol, T cells were cultured for up to 12 weeks continuously. At the end of 12 weeks, the cells continued to proliferate. The limiting factor in further longterm maintenance, however, was the availability of autologous accessory cells as feeder cells. Human subject protocols were approved by the UCSD Committee on Human Subjects.

EBV-TRANSFORMED B CELL LINES

Epstein Barr virus transformed B cell lines (EBVB) were initiated by culturing PBMC at a concentration of 1×10^6 /ml in 96-well flat bottomed tissue culture plates (Costar) with cyclosporin A 2 µg/ml and supernatant from the EBV-shedding line 1437, as previously described.¹³ Histocompatibility locus antigen typing of EBVB was done using a standard microcytotoxicity assay.

Table 1 Antibodies used in these studies

<i>Cluster</i>		<i>Antibody designation</i>	<i>Predominant cell type expressing marker</i>
OKT11	CD2		T cells
Leu 4	CD3		Mature T cells
Leu 3a	CD4		Helper/inducer T cell subset
Leu 2a	CD8		Suppressor/cytotoxic T cell subset
Leu 11b	CD16		NK cells and some cells that mediate antibody dependent cellular cytotoxicity
Leu 7			NK cells and some cytotoxic T lymphocytes
Leu 19			NK cells and some cytotoxic T lymphocytes

CELL SURFACE MARKERS ON T CELL LINES

Cell surface markers on lymphocytes were determined using the murine monoclonal antibodies Leu 2a, Leu 3a, Leu 4, Leu 7, Leu 11b, Leu 19 (Becton Dickinson, Mountain View, CA), and OKT11 (ATCC). The cluster designation (CD) determinants recognised by these antibodies and the cells on which they are found are listed in Table 1. Briefly, cells were incubated for 45 minutes at 4°C in the presence of murine monoclonal antibody, washed in phosphate buffered saline and then incubated for 45 minutes with fluorescein-isothiocyanate (FITC)-conjugated rabbit antimouse immunoglobulin (Zymed, So, San Francisco, CA). The per cent of positive staining cells was determined by flow cytometry using an Ortho Cytofluorograph system 50-H (Ortho Diagnostics, Westwood, MA) counting 10000 cells. The variability of this assay is less than 5% for triplicate samples.

PROLIFERATION

T cells were collected 10 days after the last stimulation with mitogen. Proliferation assays were carried out in triplicate in 96-well U-bottomed plates in 200 µl volumes in complete medium containing 10% FCS. For the mixed lymphocyte reaction (MLR), 2×10^4 viable responder T cells/well were incubated with either 2×10^4 autologous or allogeneic irradiated (10000 R) EBVB or 10^5 irradiated PBMC (3000 R) as stimulator cells. For mitogen stimulation, 2×10^4 T cells together with 10^5 irradiated (3000 R) PBMC as feeder cells were stimulated with either PHA 20 µg/ml, Con A 2 µg/ml or both. Studies were also performed in the presence of 1 µg/ml indomethacin. Cells were incubated for 48 h in 5% CO₂ at 37°C and then pulsed with 0.5 µCi ³H-thymidine/well. Cells were harvested 24 h later onto glass fibre filters using an automatic cell harvester (MASH, Microbiological Assoc Inc, Bethesda, MD), after which filters were counted in a liquid scintillation counter.

IL-2 ASSAY

T lymphocytes, 10^6 /ml, were stimulated in the

absence of accessory cells in U-bottomed microtitre plates with PHA 20 µg/ml, PMA 25 ng/ml, or a combination of PHA and PMA. After 24 h, supernatants were harvested, two-fold serially diluted, and assayed for IL-2 using the IL-2 dependent murine cell line NK, as previously described.^{14,15} One unit of IL-2 was defined as the reciprocal of the dilution of IL-2 resulting in a proliferative response which was 50% of the maximal response of 4×10^5 murine NK cells. Supernatant from the gibbon lymphoma line MLA 144, which constitutively secretes IL-2, was used as a positive control. Variability of a single control run in triplicate is consistently less than 15% in this assay. Day to day variability is higher; consequently, all samples were run simultaneously.

$$\% \text{ specific lysis} = \frac{\text{cpm release experimental group} - \text{cpm spontaneous release}}{\text{cpm after detergent lysis} - \text{cpm spontaneous release}} \times 100$$

CELL DEPLETION

To deplete Leu 7 and Leu 11b bearing cells from small intestinal lymphocyte lines, cells were incubated for 45 minutes at 4°C with Leu 7 and Leu 11b monoclonal antibodies, washed twice, and then incubated at 37°C for 45 minutes with a 1:10 dilution of guinea pig complement (Pel Freeze, Brown Deer, WI). Control cell lines were treated with complement alone.

Results

T lymphocyte lines were grown successfully from each of the six consecutive individuals from whom duodenal biopsy specimens were obtained. After one to two days in culture, blast transformation and mononuclear cell migration out from tissue fragments was noted (Fig. 1). Examination of individual biopsy fragments for cell viability by acridine orange and ethidium bromide staining indicated cell variabilities ranging from 60–80%. After 10 days, greater than 1.5×10^6 mononuclear cells could be harvested from two biopsies. Subsequent doubling time of these cells in culture was approximately five days. Data referring to phenotype include data from patients with coeliac disease. All functional data, however, refer to non-coeliac cell lines.

CELL PHENOTYPE

After six to 12 weeks in culture, small intestinal lymphocytes were predominantly CD2, CD3, and CD4 positive (Table 2). With the exception of line ALS, the intensity of CD8 staining was considerably less than that of CD4. One to 7% of cells expressed Leu 7 or CD16. Cell line ALS had the highest proportion of Leu 7 and CD16 positive cells, and a

CYTOTOXICITY

T lymphocytes were used 14 days after last exposure to mitogen and nine days after last exposure to interleukin 2. Target cells were radiolabelled by incubation at 37°C in 5% CO₂ for one hour in PBS containing 1 mCi/ml ⁵¹Cr sodium chromate (New England Nuclear Corp, Boston, MA). K562 was used as a human NK sensitive target. Raji and Daudi cell lines were used as NK resistant targets. Other targets included allogeneic and autologous EBVB prepared in our laboratory. Effector cells (5×10^5), or dilutions, were added in a 100 µl volume to 96-well U-bottomed microtitre plates after which 10^4 ⁵¹Cr-labelled targets in 100 µl were added. After incubation for four hours at 37°C in 5% CO₂, specific lysis was calculated using the formula:

majority of ALS cells, expressed Leu 19. Some variability in phenotype was observed. This was particularly noted in the line ALS in which a large proportion of cells expressed CD8 at 12 weeks. It is worth noting, however, that with the exception of ALS, this protocol avoids excessive expansion of IL-2 dependent cytolytic cells.

CYTOTOXICITY

All four lines tested were cytotoxic for K562, and NK sensitive target line. ALS demonstrated substantial NK activity whereas, in three other lines, levels of cytotoxicity were low, but titrated with decreasing effector to target cell ratios (Fig. 2a). Further, in the two cases tested, cytotoxicity for K562 was abrogated by the depletion of Leu 7 and Leu 11b positive cells (Fig. 2b). Daudi, an NK resistant cell line, was not killed by the intestinal cell lines (Fig. 2c), nor were the NK resistant cell line Raji or autologous or allogeneic EBV transformed B cells (data not shown).

Table 2 Phenotype of intestinal T lymphocyte lines

Cell line	Duration in culture (wk)	Cells expressing marker (%)						
		CD2	CD3	CD4	CD8	Leu 7	CD16	Leu 19
1 AH	6	89	90	90	31	3.7	2.1	NT
2 PR	6	91	97	84	28	2.7	1	NT
	12	NT	89	75	47	2	1	6
3 EJ	6	83	90	62	44	4	2	NT
	12	NT	93	82	10	3	2	7
4 BH	6	NT	NT	71	35	NT	NT	NT
5 ROPE	6	NT	NT	82	26	NT	NT	NT
6 ALS	6	82	93	56	55	7.2	7	NT
	12	NT	89	18	85	NT	NT	60

NT=not tested.

Fig. 1 Cell migration from a biopsy fragment after two days in culture with PHA, ConA and indomethacin. Clumps of blast cells (arrows) can be observed by phase contrast microscopy.

PROLIFERATION

Small intestinal lymphocytes proliferated in response to Con A or PHA. Further, the addition of indomethacin had little affect on either Con A or PHA stimulated proliferation of the cell lines. The addition of indomethacin to cultures, however, stimulated with both Con A and PHA resulted in a marked augmentation of thymidine incorporation (Fig. 3). In most cases, the T cell lines proliferated little if at all when HLA typed autologous EBVB, allogeneic EBVB (Table 3) or PBMC (data not shown) were used as stimulators in the MLR. Thus, no stimulation was seen in response to five of seven autologous or allogeneic EBVB, while low levels of proliferation were detected with EBVB stimulators from AH and YE which shared only HLA A24. The HLA typing of the cells is indicated in Table 4.

IL-2 SECRETION

Of four cell lines tested, all secreted IL-2 in response to a combination of PMA and PHA (Table 5). None secreted IL-2 in response to PMA alone. The ALS line, which contained a low proportion of CD4 cells

Table 3 Proliferation of intestinal T cell lines in response to autologous and allogeneic stimulator cells

	Source of autologous or allogeneic EBV transformed B cells						
	Subject						
	AH	RP	ALS	YE	WJG	DN	GMH
Source of T cell line							
AH	621	0	0	2483	0	0	0
PR	724	0	0	3842	0	0	0
ALS	0	0	0	0	0	0	0
EJ	1842	0	0	4410	0	0	0

2 × 10⁴ T cells/well were cultured with 2 × 10⁴ irradiated autologous or allogeneic EBVB as described in Methods. Results are expressed as cpm ³H-thymidine incorporated with backgrounds subtracted. Backgrounds for cultures containing T cells alone ranged from 68 to 330 cpm and backgrounds for cultures containing EBV transformed B cells alone range from 246 to 1356. Results are means of triplicate cultures. No proliferation was seen when the same T cells were stimulated with irradiated (3000 R) autologous or allogeneic PBMC. In controls, AH, EJ, PR, and ALS T cells stimulated with PHA alone (10 µg/ml) yielded 8375, 15 573, 12 457, and 18 010 cpm respectively.

Fig. 2 (a) Specific cytotoxicity of four intestinal lymphocyte lines for K562, and NK sensitive target cell line at varying effector: target ratios. (b) Specific cytotoxicity of lines AH and EJ for K562, the NK sensitive target, before and after depletion of Leu 7 and Leu 11b (CD 16) cells. (c) Specific cytotoxicity of AH and EJ cell lines for K562 and for the NK resistant target, DAUDI. Similar results were obtained using the NK-resistant cell line, Raji, and EBV transformed B lymphoblasts as targets (data not shown).

(Table 2), secreted little IL-2. It is notable that these experiments were performed in the absence of accessory cells.

Discussion

Methods for the longterm cultivation of T lymphocyte lines from human small intestine will provide a powerful tool to study the intestinal immune system

and its role in disease. Using mitogen stimulated proliferation of lymphocytes in small intestinal endoscopic biopsies, we have been able to develop several T cell lines which can be maintained in culture for at least 12 weeks. As intraepithelial T lymphocytes are predominately CD8 positive, and fail to proliferate in response to mitogens,^{16,17} it is probable that the majority of lymphocytes in this culture system originate from the lamina propria. This is supported by the finding that five of six of the T cell lines contained predominantly CD4 positive T lymphocytes, a phenotype that predominates in the lamina propria.¹⁻⁴

Fig. 3 Tritiated-thymidine incorporation in T cell lines stimulated with PHA 20 µg/ml, Con A 2 µg/ml or a combination of PHA and ConA. Solid bars are cultures without indomethacin and hatched bars are cultures with indomethacin 1 µg/ml. Data are expressed as CPM and horizontal bars represent the SEM. Proliferation induced by PHA and ConA combined is significantly augmented by indomethacin ($p < 0.05$; Mann Whitney one-tailed test).

Table 4 HLA type of donors of responder and stimulator cells

Subject	A	B	DR	DO
AH	3 24	7 35	1 2	W1 -
PR	1 11	8 35	1 3	ND
ALS	2 -	8 -	3 5	W3 -
EJ	3 28	14 59	6 -	ND
YE	1 24	8 -	3 3	W2 W2
WJG	2 11	7 44	2 7	W1 W2
GMH	2 -	5 39	2 6	W1 -
DN	3 11	7 8	ND	ND

ND - not determined; HLA DQ types were determined by microcytotoxicity, and for some lines by restriction fragment length polymorphism analysis.

Table 5 IL-2 secretion by intestinal T cell lines

T cell line	Stimulus		
	PHA 20 µg/ml	PMA 25 ng/ml	PHA (20 µg/ml) + PMA (25 ng/ml)
AH	0	0	66
PR	0	0	70
ALS	0	0	2
EJ	5	0	26

IL-2 secretion is expressed as units/ml.

T cell lines have been developed previously from other tissues, including kidney,^{18,19} liver,²⁰ cerebrospinal fluid,²¹ and skin.^{22,23} In several cases, mitogens have been used^{20,24} to expand such cell populations. Our strategy was to avoid IL-2 stimulation at the initiation of culture because it might result in the selective expansion of populations of IL-2-responsive non-MHC restricted cytolytic cells.²⁵ In contrast, the use of PHA to expand T cell populations does not appear to interfere with the ability to subsequently develop antigen specific helper T lymphocyte lines.²⁶⁻²⁸ Con A stimulation was used here, however, may induce low levels of CD8 expression on CD4 positive cells,²⁹ resulting in a dual positive subpopulation.

The definition of NK cells and their distinction from lymphokine-activated killer cells (LAK) has been controversial.³⁰⁻³³ Lanier *et al* have suggested that the term 'NK cell' be applied to a specific cell lineage lacking CD3 and expressing CD16.³⁴ Others have suggested, however, that all cells that are cytotoxic for NK sensitive targets should be labelled 'NK cells'.³⁵ In some studies, neither cells expressing NK markers³⁹ nor cells exhibiting NK cytolytic activity^{9,12} were found in intestinal lymphocytes. Leu 7, Leu 11, and Leu 19 positive cells, however, have been reported in the intestine in other studies.^{4,36,37} Moreover, low but consistent killing of NK-sensitive targets has been demonstrated using freshly isolated intestinal lymphocytes at a high lymphocyte:target ratio. Whichever definition of NK cells is used, the current studies show small numbers of cells expressing phenotypic markers associated with NK cells and low levels of NK killing in lymphocyte lines derived from the small intestine. Finally, we note that in peripheral blood, a small population of CD3+ Leu 19+ cells can mediate non-MHC restricted cytotoxicity.³³ It may be that the high levels of K562 killing with ALS reflect cells with that phenotype in the ALS line.

The CD4 positive small intestinal lymphocyte lines developed herein secreted IL-2, consistent with their role as T helper cells. In this regard, two classes of murine T helper cells can be distinguished based on

the lymphokines they produce.³⁷ One class, termed type 1 T helper cells, produces IL-2 and gamma interferon, whereas the second class termed type 2 T helper cells, produces IL-4 and IL-5.^{15,37} As IL-5 alone and in combination with IL-4 is important in regulating the expression of the IgA class,¹⁵ it will be important to further characterise the lymphokine products of the T helper cell populations in the small intestinal mucosa.

Two points warrant comment regarding the growth and proliferation of the small intestinal T cell lines. First, the addition of indomethacin to Con A and PHA stimulated small intestinal T cell lines augmented their proliferation as much as two-fold, suggesting that it significantly augments the growth of these cell lines by inhibiting prostaglandin E2 secretion.^{27,38} Second, the small intestinal T cell lines exhibited little or no proliferation in an autologous or allogeneic MLR, whereas freshly isolated intestinal mononuclear cells have been reported to proliferate in a MLR.³⁹ We have not explored the mechanism responsible for this difference. It is clear, however, at a technical level, that our method and those used to isolate cells from surgical specimens differ markedly.

Tissue derived lines have provided important functional information regarding local T lymphocyte function. T cell lines derived from the skin lesions of individuals with lepromatous leprosy suppressed mitogen driven T lymphocyte proliferation in an antigen specific HLA restricted fashion.²² T cell lines derived from individuals with tuberculoid leprosy do not show suppression, suggesting a polarisation in the immune response at the level of the T cell. T cell lines derived from renal allograft biopsies are cytolytic for donor targets.^{18,19} This cytotoxicity is HLA restricted. Finally, in studies done using T cell lines derived from individuals with hepatitis B, antigen specific help for the production of antibodies to hepatitis B core antigen was observed.³⁹ In diseases in which a specific antigen is implicated, T cell clones may be useful for mapping relevant antigen determinants.^{26,40,41,43} The ability to culture human intestinal T lymphocytes should permit the characterisation in more detail of the normal phenotypic and functional properties of these cells. Furthermore, the ability to utilise cells derived from endoscopic biopsies should permit a fuller understanding of the immunological mechanisms involved in intestinal disease in patients who are not undergoing surgical resection.

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