Isolation and characterization of lymphocyte-like cells from a lamprey

Werner E. Mayer*, Tatiana Uinuk-ool*, Herbert Tichy*, Lanier A. Gartland†, Jan Klein*, and Max D. Cooper†‡

*Max-Planck-Institut für Biologie, Abteilung Immungenetik, Corrensstrasse 42, D-72076 Tübingen, Germany; and †Howard Hughes Medical Institute, University of Alabama, 378 Wallace Tumor Institute, Birmingham, AL 35294

Contributed by Max D. Cooper, August 30, 2002

Lymphocyte-like cells in the intestine of the sea lamprey, *Petromyzon marinus*, were isolated by flow cytometry under light-scatter conditions used for the purification of mouse intestinal lymphocytes. The purified lamprey cells were morphologically indistinguishable from mammalian lymphocytes. A cDNA library was prepared from the lamprey lymphocyte-like cells, and more than 8,000 randomly selected clones were sequenced. Homology searches comparing these ESTs with sequences deposited in the databases led to the identification of numerous genes homologous to those predominantly or characteristically expressed in mammalian lymphocytes, which included genes controlling lymphopoiesis, intracellular signaling, proliferation, migration, and involvement of lymphocytes in innate immune responses. Genes closely related to those that in gnathostomes control antigen processing and transport of antigenic peptides could be ascertained, although no sequences with significant similarity to MHC, T cell receptor, or Ig genes were found. The data suggest that the evolution of lymphocytes in the lamprey has reached a stage poised for the emergence of adaptive immunity.

Vertebrates with jaws (gnathostomes) possess an adaptive (anticipatory) immune system that is absent in nonvertebrates (1). The hallmark of the system is its capacity to anticipate an encounter with any protein or carbohydrate foreign to the body and to retain a memory of this encounter. This memory enables the organism to respond in an accelerated manner after reexposure to the same antigenic stimulus. The adaptive immune system is comprised of a complex array of different cell types and molecules intricately interacting with one another. In this array, three cell types and three types of molecules are central to the immune-system function. These include T and B lymphocytes as well as the dendritic cells, all derived from a common hemopoietic stem cell (2), and the T cell receptor (TCR), B cell receptor (BCR, Ig), and MHC molecules. Lymphocytes of jawed vertebrates are small cells in which the nucleus, characterized by a densely compacted, coarse chromatin, occupies ≈90% of the cell volume, and the cytoplasm is limited to a thin rim (3). They possess the ability to respond to stimuli by enlarging their volume (lymphoblast transformation) and dividing repeatedly. Although some of the activated lymphocytes terminally differentiate into effector cells, others return to the state of small, resting cells (1). The proliferative phase multiplies those lymphocytes that express TCR or Ig receptors specific for the stimulating antigen and thus expands them into clones of cells that express the same receptor. The return to the morphology of small lymphocytes marks the generation of memory cells. The differentiated lymphocytes can kill infected target cells (cytotoxic T lymphocytes), assist other cells in their development (helper T lymphocytes), or become Ig (antibody)-secreting plasma cells (B lymphocytes).

TCR, Ig, and MHC receptors have been found thus far only in jawed vertebrates. Attempts to demonstrate their presence in living jawless vertebrates, represented by lampreys and hagfishes, have failed (4). On the other hand, small cells with darkly staining nuclei and scanty cytoplasm have been observed in histological sections prepared from either lamprey or hagfish tissues (5). Because of the failure to obtain clear evidence for the existence of the adaptive immune system in jawless fishes, the identity of these cells has remained in doubt. The aim of the present study was to exploit contemporary methods of cell separation to isolate the agnathan lymphocyte-like cells to characterize them morphologically and by the genes they express.

Materials and Methods

Source and Preparation of Cell Suspension. Ammocoete larvae (8–13 cm long) of the sea lamprey, *Petromyzon marinus* (from Lake Huron, MI) were dissected along the ventral side to extract the intestine and the associated typhlosole (spiral valve). Cells harvested by maceration of these tissues between two glass slides were suspended in one part water and two parts PBS. Lamprey intestinal cells with forward and sideward light-scattering characteristics of a control population of intraepithelial lymphocytes from the mouse intestine were identified and sorted by using the MoFlo cytometer (Cytomation, Fort Collins, CO). Sorted cells were prepared for electron microscopy by fixation with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h before centrifugation and resuspension in the same buffer. For light-microscopic evaluation, sorted cells were coated onto glass slides by using a cytocentrifuge, stained with Wright stain, washed, and photographed.

Construction of cDNA Library. Total RNA was extracted from 1×10^6 sorted cells with the help of the RNeasy mini kit (Qiagen, Hilden, Germany). To construct a directional cDNA library with high representation of 5′ ends, the SMART cDNA library-construction kit (BD Biosciences/CLONTECH, Heidelberg, Germany) was used according to manufacturer protocol. Briefly, ≈200–300 ng of total RNA were reverse-transcribed by the PowerScript reverse transcriptase (BD Biosciences/CLONTECH), and the transcript was subjected to cDNA amplification in 25 cycles of long-distance PCR. The cDNA was size-fractionated by chromatography on CHROMA SPIN-400 columns, and fractions containing cDNA larger than 500 bp were pooled and directionally inserted into the λTriplEx2 vector. Ligated DNA was packaged into phage particles by using the Gigapack Gold *in vitro* packaging kit (Stratagene, Amsterdam) and transfected into the *Escherichia coli* strain XL1-Blue (BD Biosciences/CLONTECH), which then were plated. A total of 2×10^6 independent plaque-forming units was obtained. Not all inserts represented full-length cDNA sequences, presumably because of incomplete DNA synthesis and preferential amplification of small molecules in the PCR step used to increase the yield of cDNA. Insert sizes ranged from 500 bp to 4 kb, the majority being between 500 bp and 1.5 kb.

Analysis of Individual Clones. The unamplified library was plated at a dilution yielding single, well isolated plaques. A total of 9,312 clones was picked manually and resuspended in 500 μl of lambda dilution buffer (100 mM NaCl/10 mM $MgCl_2$/35 mM Tris·HCl,

Abbreviations: TCR, T cell receptor; BCR, B cell receptor.

‡To whom correspondence should be addressed. E-mail: max.cooper@ccc.uab.edu.

 www.pnas.org/cgi/doi/10.1073/pnas.212527499

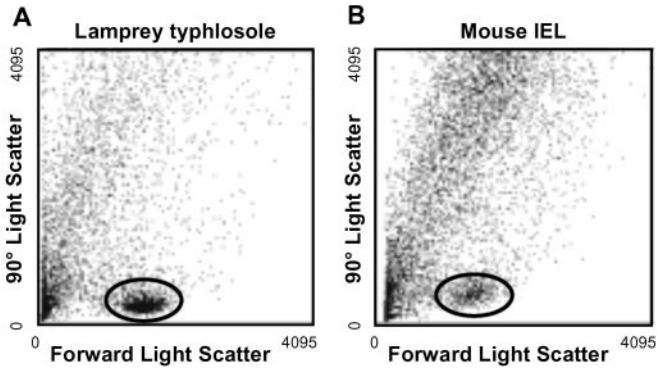

Fig. 1. Fractionation of lamprey intestine cells by light-scattering characteristics using flow cytometry: forward (180°) and sideward (90°) light-scatter diagrams of lamprey typhlosole cells (*A*) and mouse intestinal intraepithelial lymphocytes (IEL, *B*). The populations containing small lymphocyte-like cells are encircled.

pH 7.5/0.01% gelatin). Insert DNA from 1 μl of the phage suspension was PCR-amplified in a 25-μl reaction volume with the primer pair TriplEx2LD5 (5′-CTCGGGAAGCGCGCCATTGTGTTGGT-3′) and TriplEx2LD3 (5′-ATACGACTCACTATAGGGCGAATTGGCC-3′) by either HotStar *Taq* polymerase (Qiagen) or the Expand long-template PCR system (Roche Diagnostics) using the manufacturer's buffer systems. Cycling conditions in a PTC-200 Peltier thermal cycler (MJResearch and Biozym, Hessisch Oldendorf, Germany) were: initial denaturation at 94°C for 2 min (Expand long-template PCR system) or 15 min (HotStar *Taq* polymerase), followed by 10 cycles of denaturation at 94°C for 10 sec, primer annealing at 65°C for 30 sec, and elongation at 68°C (Expand long-template PCR system) or 72°C (HotStar *Taq* polymerase) for 6 min. Twenty more PCR cycles then were added under the same conditions except for an incremental increase of 20 sec in the elongation step of each cycle. The PCR was completed by a final elongation for 7 min at 72°C. The PCR products were purified and subjected to single-pass sequencing by the custom-sequencing service of MediGenomix (Martinsried, Germany) using the pTripl5Seq2 (5′-GAAGCGCGCCATTGTGTT-3′) primer annealing next to the 5′ end of the insert cDNA. The sequences varied in length and quality, with useful sequences generally not exceeding 800 bp. Of the 9,312 sequences, 8,043 judged to be of high quality and free of di- or trinucleotide repeats were chosen for further analysis.

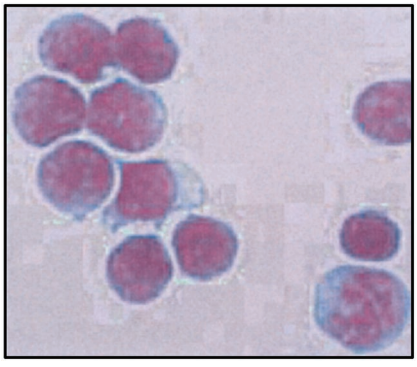

Fig. 2. Light-microscopic view of sorted lamprey lymphocyte-like cells stained with Wright stain. The large, darkly stained nuclei and the thin rim of agranular cytoplasm is highly reminiscent of mammalian lymphocytes.

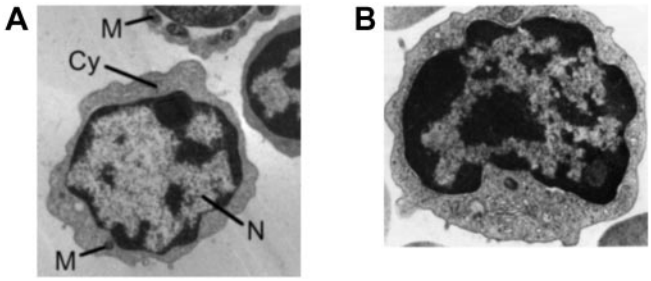

Fig. 3. Transmission electron-microscopic views of lamprey lymphocyte-like cells (*A*) and mouse intestinal epithelial lymphocytes (*B*). The cells contain large nuclei (N) with heterochromatin forming a peripheral rim adjacent to the nuclear envelope. The nuclei are surrounded by a thin layer of cytoplasm (Cy) containing several small spherical mitochondria (M).

Sequence Analysis. Insert sequences were subjected to BLASTX searches (6) against the nonredundant protein database at the National Center for Biotechnology Information (NCBI). Immunologically relevant sequence matches to database entries were examined in more detail. By using the NCBI BLASTCLUST program, the sequences were grouped into clusters of identical or overlapping sequences. In this step, 1,735 sequences were combined into clusters containing between 2 and 210 sequences.

Results and Discussion

Physical Characteristics of Lamprey Lymphocyte-Like Cells. The typhlosole, an invaginated spiral valve spanning the length of the lamprey intestine, was the primary tissue source for the cells in this analysis. Cells derived from this tissue were analyzed in parallel with mouse intestinal epithelial cells by automated flow cytometry. The lamprey typhlosole contains a discrete subpopulation of cells with light-scatter characteristics that closely resemble those of the lymphocytes in the mouse intestinal epithelium (Fig. 1). This population of lamprey cells was isolated with an automated cell sorter for further analysis. Both light (Fig. 2) and electron (Fig. 3) microscopy reveal the purified lamprey cells to represent a fairly homogeneous population in terms of size, staining properties, and structure. The diameter of the cells ranges from 6 to 9 μm, mainly because of variation in the amount of cytoplasm present. Most of the cell volume is taken up by the darkly staining nucleus, the scanty, pale cytoplasm often being limited to a narrow rim in the light-microscopic preparations. In electron-microscopic preparations the nucleus is quite dense, filled with compacted chromatin. The cytoplasm is rich in ribosomes, both free and attached to the rough endoplasmic

Table 1. Classification of ESTs according to BLASTX search results

Similarity group	Frequency
Mitochondrial-derived RNA	0.032
Ribosomal proteins	0.260
Signal transduction-associated proteins	0.019
Calcium-binding proteins	0.007
Retroelement-associated proteins and transposons	0.031
Ion channels and transporters molecules	0.003
Chaperones	0.034
Nuclear DNA-associated proteins	0.013
Transcription factors	0.009
Translation factors	0.020
Cell proliferation and differentiation-associated proteins	0.010
Structural proteins	0.020
Metabolic proteins	0.057
Other proteins	0.090
Ferritin	0.043
Proteins without significant similarity	0.349

Table 2. Results of BLASTX searches: Catalogue of proteins expressed in hematopoietic and lymphocyte-like cells

Clone	Similarity to	Species	Accession no.	Function
Genes controlling lymphocyte ontogeny				
	Spi transcription factor	*Petromyzon marinus*	AAF78904	Transcription factor
	Ikaros transcription factors	*Petromyzon marinu*	AAL67302/AAL62094	Transcription factors
42A10	Mast/stem cell growth factor receptor (tyrosine-protein kinase c-Kit)	*Gallus gallus*	Q08156	Tyrosine protein kinase
38A03	Uncharacterized hematopoietic stem/progenitor cells protein MDS003	*Homo sapiens*	NP_060938	Unknown
Genes involved in lymphocyte signaling				
26A03	Human leukocyte common antigen CD45	*Homo sapien*	NP_002829	Protein-tyrosine phosphatase
25F12	B cell phosphoinositide 3-kinase adaptor (BCAP)	*Mus musculus*	NP_113553	Links BCR-associated kinases with phosphatidylinositol 3-kinase
51E10	CD3 ε associated protein; antisense to ERCC-1 (CAST)	*Homo sapiens*	NP_036231	T-cell activation
11C05	Activated protein kinase C receptor (RACK1)	*Rattus norvegicus*	A36986	PRKC-mediated signaling
12D11	Thymosin β-10	*Rattus norvegicus*	AAB37101	Actin monomer sequestering protein
79D04	IgG Fc-binding protein	*Homo sapiens*	AAD39266	Role in intestinal immune protection and inflammation
O8F12	c-Kit-related kinase	*Xenopus laevis*	I51703	Tyrosine protein kinase
43B05	Kit-like A	*Lethenteron reissneri*	BAA84745	Tyrosine protein kinase
14C11	Tumor necrosis factor receptor superfamily member	*Mus musculus*	NP_035739	Binding of tumor necrosis factor
18A11	Progesterone receptor-related protein p23	*Gallus gallus*	B56211	Chaperone, disrupting receptor-mediated transcriptional activation
18B09	Ran GTP-binding protein	*Xenopus laevis*	BAA89696	GTP-binding protein
30G05	Protein tyrosine phosphatase	*Mus musculus*	CAA76755	Protein tyrosine phosphatase
34A04	fms-related tyrosine kinase 3	*Homo sapiens*	XP_012297	Tyrosine kinase
34E11	Myc-binding protein	*Xenopus laevis*	I51586	Myc binding, regulation of gene expression during differentiation
36G05	ATP-stimulated glucocorticoid-receptor translocation promoter	*Rattus norvegicus*	NP_077357	Increases binding of activated glucocorticoid-receptor to nuclei
48H11	Lanthionine-synthetase component C-like protein-coupled receptor	*Homo sapiens*	NP_061167	G protein-coupled receptor
58B05	Granalcin	*Mus musculus*	AAM66720	Calcium binding
11D11	Calmodulin 2	*Homo sapiens*	NP_001734	Calcium binding
06F07	Calcium-dependent protein kinase SK5	*Glycine max*	P28583	Calcium-dependent signal transduction
Genes required for lymphocyte proliferation and migration				
09D03	CD98	*Homo sapiens*	XP_043189	Amino acid transporter
87B05/78D10	CD9/CD81 (target of antiproliferative antibody 1)	*Bos taurus* *Rattus norvegicus*	P30932 NP_037219	Tetraspanin receptors, participation in cell migration and adhesion
Genes encoding proteasomal subunits and an ABC transporter				
04A09	ABCB9	*Homo sapiens*	NP_062571	ABC transporter
55B12	PSMB4	*Xenopus laevis*	P28024	Protein degradation
93B06	PSMB7	*Homo sapiens*	AAH00509	Protein degradation
97D07	26S proteasome subunit pUb-R3	*Mus musculus*	BAA97575	Protein degradation
66F06	Proteasome subunit α type 2 (proteasome component C3)	*Xenopus laevis*	P24495	Protein degradation
23B08	Proteasome subunit α type 6	*Mus musculus*	NP_036098	Protein degradation
97G10	Proteasome inhibitor PI31 subunit	*Homo sapiens*	NP_006805	Protein degradation
Other immunologically relevant genes				
06D11	Chaperone protein dnaJ	*Haemophilus ducreyi*	P48208	Chaperone
34G10	Heat-shock 90-kDa protein 1, β	*Homo sapiens*	XP_018115	Chaperone
29C10	Heat-shock 90-kDa protein 1, α	*Homo sapiens*	NP_005339	Chaperone
34D12	Heat-shock cognate 70 protein	*Gallus gallus*	CAA06233	Chaperone
73H07	dnaK-type molecular chaperone hsp70	*Pleurodeles waltl*	JC5642	Chaperone
84F09	Heat-shock protein hsp70-related protein	*Homo sapiens*	XP_028530	Chaperone
97B07	Heat-shock protein 86	*Homo sapiens*	AAA36024	Chaperone
87A09	Macrophage migration inhibitory factor	*Gallus gallus*	Q02960	Regulation of macrophage function in inflammation
25B03	CXC chemokine receptor 4	*Acipenser ruthenus*	CAB60252	Directs migration of leukocytes in lymphoid organs
15G09	Cathepsin L	*Myxine glutinosa*	AAF19630	Cysteine protease
73A07	Cystatin B thiol proteinase inhibitor	*Homo sapiens*	NP_000091	Cysteine protease inhibitor
92D12	Egg-white cystatin; cystatin F; leukostatin	*Gallus gallus* *Homo sapiens*	P81061 NP_003642	Cysteine protease inhibitor
97A10	Lysosomal trafficking regulator	*Mus musculus*	NP_034878	Lysosomal traffic
15E08	Lymphocyte antigen 6 complex-related	*Mus musculus*	NP_034872	GPI-linked cell-surface glycoprotein
26C02	Complement component 1, q subcomponent-binding protein	*Mus musculus*	NP_031599	Multifunctional protein; regulation of complement system
44BG01	Hepsin	*Homo sapiens*	NP_002142	Blood coagulation, fibrinolysis, complement activation

Table 2. (continued)

Clone	Similarity to	Species	Accession no.	Function
70H12	Glutathione-dependent hematopoietic prostaglandin D synthase	*Gallus gallus*	O73888	Conversion of prostaglandin PGH2 to PGD2
49B02	Zygin 2; fasciculation and elongation protein ζ 2; Pre-T/NK cell-associated protein (3C1)	*Homo sapiens*	NP_005093	Axonal fasciculation in neural tissue
13G06	Mitogen-inducible gene 2 (mig-2) or talin	*Homo sapiens*	XP_051693	Connects cytoskeletal structures to plasma membrane
40A01	Ribosome-associated membrane protein 4 (RAMP4)	*Rattus norvegicus*	CAB40910	Controls glycosylation of MHC class II-associated invariant chain
29A06	Tumor necrosis factor-inducible protein TSG-6, (hyaluronate-binding protein PS4)	*Oryctolagus cuniculus*	P98065	Hyaluronan-binding protein
11B12	Interferon-inducible protein 16	*Rattus norvegicus*	NP_110460	Unknown
11E10	Slit	*Danio rerio*	AAG36772	Inhibits leukocyte chemotaxis
92F05	Dentritic cell protein DC6	*Homo sapiens*	NP_064574	Unknown
46F05	RW1 protein	*Mus musculus*	AAC15232	Possible role in antiviral immune response
58A07	ADP-ribosylation-like factor-6 interacting protein (ARL6)	*Homo sapiens*	Q15041	Role in hematopoietic maturation
62D09	Putative human HLA class II-associated protein	*Homo sapiens*	NP_006296	Binding of cytoplasmic domain of the HLA DR-α chain
17B05	Thioredoxin	*Oryctolagus cuniculus*	P08628	Redox reactions, augments expression of IL-2 receptor
72D07	Thioredoxin peroxidase 1	*Rattus norvegicus*	NP_058865	Immunoregulation of natural killer cell activity
25D08	Peroxidasin	*Drosophila melanogaster*	AAF47668	Extracellular matrix-associated peroxidase in hemocytes
75F03	Peptidyl-prolyl *cis-trans*-isomerase (cyclophilin D)	*Mus musculus*	NP_032046	Binds cyclosporin A
83G05	B cell translocation gene-2 (BTG2), antiproliferative	*Mus musculus*	NP_031596	Cell-cycle progression
84A11	Tumor protein, translationally controlled 1	*Homo sapiens*	NP_003286	IgE-dependent histamine-releasing factor
77H04	SEC61, γ subunit of protein translocation complex	*Mus musculus*	XP_122171	Protein translocation
09G06	Ferritin heavy chain	*Salmo salar*	P49946	Iron storage and cellular regulation
47B04	Ferritin H-3	*Oncorhynchus mykiss*	BAA13148	Iron storage and cellular regulation
91F11	Ferritin light chain 2	*Mus musculus*	NP_032075	Iron storage and cellular regulation

reticulum. The surface of most of the cells appears to be largely smooth. All these characteristics are histological hallmarks of "small lymphocytes" (7). Indeed, even experienced morphologists would be unable to distinguish the lamprey cells from mouse lymphocytes if presented with unmarked preparations of the two. Nevertheless, in the absence of sufficient information about the functional properties of the lamprey cells, we refer to them as "lymphocyte-like."

Gene-Expression Profile. The purified population of the lamprey lymphocyte-like cells was used as a source for RNA isolation, production of a cDNA library, and sequencing of randomly selected cDNA clones. In the collection of 8,043 sequences remaining after the exclusion of poor-quality sequences from the initial number of 9,312, there were 1,735 that were represented more than once in the set. Of these, sequences of 73 genes were found twice, 24 three times, and 18 four times. Some 15% of the 8,043 sequences were found in >10 copies. Most abundant in this set were mitochondrial RNA-derived sequences and rRNA, followed by a group of 178 ESTs of an unidentifiable gene, 157 sequences encoding ribosomal protein L41, and 135 ESTs of the ferritin heavy chain. The remaining clusters contained mostly heat-shock protein genes, genes encoding transcription- and translation-associated factors, as well as other housekeeping genes. The large number of unique sequences in the data set (6,308/8,043 or 78.4%) indicates that the library covered a wide range of genes expressed at low abundance. However, although the stringency of the BLASTX search was set rather low (a discrimination threshold of $E < 0.009$) so as to detect as many similarities between lamprey and mammalian genes as possible, the identity of only 2,590 of the 6,308 unique sequences ($\approx$40%) could be established. This is an indication that a fraction of the ESTs may be derived from 3′ UTRs. The remaining 60% of unidentified sequences may represent either novel ESTs or sequences with very low similarity to known genes. As would be expected for metabolically active cells, ESTs of genes involved in the control of gene expression and protein synthesis were highly represented in the set. They included 36 genes encoding transcription factors, 15 genes participating in RNA synthesis and processing, 944 ribosomal protein-encoding genes, 81 genes specifying translation factors, and 6 genes functioning in signal peptide recognition (Table 1).

Below we highlight those ESTs that are homologous to mammalian genes prominently expressed in lymphocytes and that may be relevant to immune responses. Because of space limitations, we do not describe the genes from which the ESTs are derived here, nor do we provide evidence for their orthology with mammalian genes. For five of these genes, this information is given in the accompanying paper (8); for others it will be given in publications that are in preparation. The purpose of the present survey is to provide an overview necessary for drawing conclusions about the possible involvement of the lamprey lymphocyte-like cells in immunity. For convenience, the survey is divided into several functional categories.

Genes Controlling Lymphocyte Ontogeny. The differentiation of mammalian lymphocytes is driven by a set of transcription factors that includes proteins of the Spi (9) and Ikaros (10, 11)

families. Members of both families have been demonstrated in the lamprey (12–14), and evidence for their expression in tissues rich in lymphocyte-like cells has been provided. To demonstrate the expression of Spi and Ikaros genes in these cells directly, we performed RT-PCR with lamprey Spi-B and Ikaros-specific primers by using extracted total RNA. A strong band representing the expected PCR product was present in each case but was absent when the reverse-transcription step was omitted (data not shown). The lamprey EST set also contains sequences specifying other factors less specifically involved in the control of hemopoiesis (Table 2). They are exemplified by the mast/stem cell growth factor receptor, a c-Kit tyrosine kinase-type protein of the colony-stimulating factor 1/platelet-derived growth factor receptor family (15).

Genes Involved in Lymphocyte Signaling. Antigenic stimulation of mammalian lymphocytes activates complex signaling pathways involving a characteristic array of enzymes and adaptor proteins (16, 17). The lamprey EST set contains sequences derived from genes encoding three proteins, CD45, BCAP (B cell adaptor for phosphoinositide 3-kinase), and CAST (CD3ε-associated signal transducer), the orthologs of which are essential for the activation of mammalian lymphocytes. Mammalian CD45 (leukocyte common antigen) is a receptor-type protein tyrosine phosphatase that dephosphorylates Srk-family protein tyrosine kinases associated with the TCR–CD3 complex after the engagement of the TCR by an MHC–peptide assembly. Mammalian BCAP is a protein activated by BCR-associated protein tyrosine kinases after the engagement of the BCR by an antigen (18). The activated BCAP mediates the interaction between the phosphatidylinositol 3-kinase and its substrate, phosphatidylinositol-(4,5)-biphosphate. Mammalian CAST is similarly an adaptor protein that, as the name implies, is constitutively associated with the ε chain of the TCR–CD3 complex. Upon its activation after the engagement of the TCR by the MHC–peptide assembly, it transduces the activation signal to other proteins in the pathway, leading to the activation of interleukin-2 and other genes (19). The lamprey counterparts of these three proteins are described in the accompanying paper (8). The mammalian proteins are expressed either exclusively or predominantly in lymphocytes or other cells of the hemopoietic progression.

Involvement in lymphocyte signaling also is prognosticated for the receptor for activated protein kinase C (RACK1), the lamprey homolog of which is identified by several ESTs in the set. The mammalian RACK1 is a WD motif-containing protein that binds specifically to the type I IFN receptor chain 2 (20). In addition to these molecules, the lamprey EST set also contains sequences encoding several other proteins, the mammalian homologs of which are involved in the signaling pathways shared by many different cell types including lymphocytes. These include three calcium-binding proteins, calmodulin and calmodulin-related kinase (16 ESTs), as well as grancalcin (1 EST). Mammalian grancalcin is expressed predominantly in myeloid cells, both normal and cancerous (21).

Genes Required for Lymphocyte Proliferation and Migration. The ability to respond to stimuli by growth, cell division, and migration is one of the functional characteristics of mammalian lymphocytes (1). Many genes control these activities, and the homologs of several of them are indicated by the lamprey EST set. An example of a gene expressed abundantly by mammalian lymphocytes during the proliferation phase of their cell cycle is CD98 (solute carrier family 3 member 2 or SLC3A2, also referred to as T cell-activation antigen; see ref. 22). It seems to have at least two functions. As a heavy chain of a dimeric transporter, it regulates the import of amino acids into the proliferating cells, and by its interaction with integrin adhesion receptors, it influences the stimulation of T lymphocytes and other cells. An example of a molecule that may influence lymphocyte migration is CD9, which is known to associate with integrins and a great number of other molecules. Lamprey orthologs of CD98 and CD9 have been identified in the EST set and characterized (8). Their considerable sequence similarity with their mammalian counterparts suggests that they may be functionally equivalent to the latter.

Genes Encoding Proteasomal Subunits and an ABC Transporter. Proteasomes are organelles of eukaryotic cells in which proteins are degraded into peptides (23). Stimulated lymphocytes and other immunologically active cells of jawed vertebrates possess a class of proteasomes specialized in producing peptides for loading onto newly synthesized MHC class I molecules. A distinctive feature of these ''immunoproteasomes'' is the replacement of subunits PSMB5, PSMB6, and PSMB7 of the ''housekeeping'' proteasomes by subunits PSMB8, PSMB9, and PSMB10, respectively. These last three subunits are encoded in genes that are regulated by IFN-γ (24), and they possess activities necessary for the generation of peptides fitting into the peptide-binding region of the class I molecules. The lamprey EST set contains six sequences encoding proteasomal proteins: PSMB4, PSMB7, 26S proteasome subunit pUb-R3, PSMA2, PSMA6, and PSMF1. Of these, the most interesting is the sequence related to the gene encoding the PSMB7. Analysis of the gene from which the EST is derived has revealed it to be related closely not only to PSMB7 of the housekeeping proteasomes but also to PSMB10 of the immunoproteasomes. The pair is believed to have arisen by gene duplication from a common ancestor, and therefore the lamprey gene could represent the state before the duplication. Phylogenetic analysis, however, favors the possibility that the lamprey gene might actually be PSMB10, which has not come under the control of IFN-γ as yet and therefore has been evolving more slowly than the IFN-γ-regulated genes (25).

In gnathostome lymphocytes the immunoproteasome-generated peptides are delivered to the site of class I molecule synthesis in the endoplasmic reticulum by the ATP-binding cassette transporters ABCB2 (TAP1) and ABCB3 (TAP2; ref. 26). The lamprey EST set contains an ABCB9 sequence that is related closely to these genes (T.U.-o., W.E.M., M.D.C., and J.K., unpublished results). The exact nature of this relationship is unclear. As in the case of PSMB7, the lamprey gene might represent the ancestral condition from which ABCB2 and ABCB3 could have arisen by gene duplication. But the situation again is complicated by the fact the ABCB2 and ABCB3 (but apparently not ABCB9) have also come under the influence of IFN-γ, and this event may have accelerated their evolution.

Other Immunologically Relevant Genes. In addition to these ''high-profile'' homologs of genes expressed in mammalian lymphocytes, the lamprey cDNA library yielded an assortment of somewhat less prominent but by no means less important genes participating in immunological responses mostly of the innate type (Table 2). Several highly conserved chaperone-encoding sequences have been found in the library at a frequency of 3.4%; they include Hsp90 and Hsp70/Hsc70-related genes. Cytokine-encoding genes are represented in the EST set by the macrophage-inhibitory factor (A. Sato, T.U.-o., N. Kuroda, W.E.M., N. Takezaki, R. Dongak, F. Figueroa, M.D.C., and J.K., unpublished results) and the chemokine receptor of the CXCR4 type (N. Kuroda, T.U.-o., A. Sato, I. E. Samonte, F. Figueroa, W.E.M., and J.K., unpublished results), which in mammals are expressed predominantly in lymphocytes and macrophages. Lysosomal cysteine proteases are represented in the collection by cathepsin L, which in mammalian lymphocytes plays a critical part in antigen degradation for presentation by MHC class II molecules (27). Cysteine protease inhibitors are exemplified by cystatin 7 (leukostatin), an inhibitor of the papain family pro-

teases, and cystatin B, presumably serving as a protector against cathepsin L and other proteases leaking from lysosomes. The Ly6 family of glycosylphosphatidylinositol-anchored proteins is represented by 14 ESTs. The lamprey genes seem to encode a 109-aa residue protein related to Ly6D and neurotoxin 1. The complement cascade is represented by the component C1q and hepsin, a cell membrane-associated serine protease participating in blood coagulation and fibrinolysis, and the prostaglandin system is epitomized by hemopoietic prostaglandin D2 synthase, the key enzyme in the production of the D and J series of prostanoids (28). Other genes included homologs of (*i*) zygin 2 expressed in human pre-T and natural killer cells (29), (*ii*) mitogen-inducible gene 2 (mig-2) or talin expressed in trout leukocytes and presumably involved in cell–cell contacts between lymphocytes (30), and (*iii*) stress-induced ribosome-associated protein 4 (RAMP4), which in mammalian lymphocytes interacts with nascent MHC class II-associated invariant chains (31).

Interpretation. The cell population isolated by flow cytometry from the intestine of the sea lamprey fits the description of lymphocytes by a number of criteria. First, morphologically the lamprey cells are indistinguishable from mammalian lymphocytes both at the light- and electron-microscopy levels. They also possess physical characteristics of lymphocytes, thus enabling us to isolate them from other hematopoietic lineage cells on the basis of their distinctive high-scatter characteristics. Second, the lamprey cells are abundant in tissues (intestine, typhlosole) that are believed to have been phylogenetically the locus operandi of lymphocytes (32). Third, judging from their expression of certain transcription factors, the lamprey lymphocyte-like cells are apparently of hemopoietic origin. Fourth, the cells express gene homologous to those prominently or characteristically expressed in mammalian lymphocytes. And fifth, when cultured in the presence of mitogens such as lipopolysaccharide and phytohemagglutinin, the cells respond by blast transformation and limited proliferation (L.A.G., unpublished data).

There is one major criterion that the cells do not seem to fulfill to qualify as bona fide lymphocytes: Thus far, they have shown no indication of expressing MHC, TCR, and BCR, the three molecules considered by some (33) to be the hallmark of lymphocytes. Although the initial homology searches of the EST set described in the present study identified several sequences with weak similarities to one or an other of the three cardinal markers of mammalian lymphocytes, further analysis of these clones proved the correspondence to be spurious. No clone with significant sequence similarity to any of the three genes could be authenticated. Although a negative result of this sort does not prove that the lamprey lacks MHC, TCR, and BCR genes, it increases the likelihood of this being the case. Furthermore, two other observations point in the same direction. First, far smaller sampling of cDNA libraries prepared by the same protocol from tissues of bony fishes yield sequences derived from these three genes (A. Sato and I. E. Samonte, unpublished data). This result may be regarded as a positive control for the experiment reported here. And second, all other efforts to find MHC, TCR, and BCR as well as other genes necessary for the trio's function (e.g., RAG, PSMB8, PSMB9, PSMB10, ABCB2, and ABCB3) also have been unsuccessful (4). Although close relatives of some of these auxiliary genes (e.g., PSBM10, ABCB2, and ABCB3) exist in the lamprey (ref. 25; T.U.-o., W.E.M., M.D.C., and J.K., unpublished results), they do not seem to have the same function as in mammalian lymphocytes.

It therefore may be fair to conclude at this stage that despite their morphological, physical, ontogenetic, and biochemical similarity to mammalian lymphocytes, the lamprey lymphocyte-like cells in the typhlosole are functionally not equivalent to true lymphocytes. They seem to have reached a stage in evolution in which many but not all of the molecules and mechanisms necessary for assuming the function of a key cellular element in the adaptive immune response have been established. What seems to be missing are a few critical genes (the MHC, TCR, BCR genes) and their auxiliaries. If this interpretation is correct, the introduction of these genes into the lamprey lymphocyte-like cells should convert them into genuine lymphocytes.

We thank Dr. G. Larry Gartland for help with flow-cytometric analysis and cell sorting, Ms. Jane Kraushaar for editorial assistance, and Dr. James G. Seeley (Hammond Bay Biological Station, U.S. Department of Interior) for ammocoete larvae of the sea lamprey.

1. Klein, J. & Horejsi, V. (1997) *Immunology* (Blackwell Scientific, Oxford).
2. Weissman, I. L. (1994) *Immunity* **1,** 529–531.
3. Weiss, L. (1984) *The Blood Cells and Hematopoietic Tissues* (Elsevier, New York), 2nd Ed.
4. Klein, J., Sato, A. & Mayer, W. E. (2000) in *Major Histocompatibility Complex: Evolution, Structure, and Function*, ed. Kasahara, M. (Springer, Tokyo), pp. 3–26.
5. Good, R. A., Finstad, J. & Litman, J. (1972) in *The Biology of Lampreys*, eds. Hardisty, M. W. & Potter, I. C. (Academic, London), Vol. 2, pp. 405–432.
6. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Miller, W. & Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402.
7. Bessis, M. (1977) *Blood Smears Reinterpreted* (Springer, Berlin).
8. Uinuk-ool, T., Mayer, W. E., Sato, A., Dongak, R., Cooper, M. D. & Klein, J. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 14356–14361.
9. Clevers, H. C. & Grosschedl, R. (1996) *Immunol. Today* **17,** 336–343.
10. Georgopoulos, K. (2002) *Nat. Rev. Immunol.* **2,** 162–174.
11. Cortes, M., Wong, E., Koipally, J. & Georgopoulos, K. (1999) *Curr. Opin. Immunol.* **11,** 167–171.
12. Shintani, S., Terzic, J., Sato, A., Saraga-Babic, M., O'hUigin, C., Tichy, H. & Klein, J. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7417–7422.
13. Haire, R. N., Miracle, A. L., Rast, J. P. & Litman, G. W. (2000) *J. Immunol.* **165,** 306–312.
14. Mayer, W. E., O'hUigin, C., Tichy, H., Terzic, J. & Saraga-Babic, M. (2002) *Scand. J. Immunol.* **55,** 162–170.
15. Sasaki, E., Okamura, H., Chikamune, T., Kanai, Y., Watanabe, M., Naito, M. & Sakurai, M. (1993) *Gene* **128,** 257–261.
16. Kane, L. P., Lin, J. & Weiss, A. (2000) *Curr. Opin. Immunol.* **12,** 242–249.
17. Kurosaki, T. (2002) *Curr. Opin. Immunol.* **14,** 341–347.
18. Okada, T., Maeda, A., Iwamatsu, A., Gotoh, K. & Kurosaki, T. (2000) *Immunity* **13,** 817–827.
19. Yamazaki, T., Hamano, Y., Tashiro, H., Itoh, K., Nakano, H., Miyatake, S. & Saito, T. (1999) *J. Biol. Chem.* **274,** 18173–18180.
20. Croze, E., Usacheva, A., Asarnow, D., Minshall, R. D., Perez, H. D. & Colamonici, O. (2000) *J. Immunol.* **165,** 5127–5132.
21. Boyhan, A., Casimir, C. M., French, J. K., Teahan, C. G. & Segal, A. W. (1992) *J. Biol. Chem.* **267,** 2928–2933.
22. Devés, R. & Boyd, C. A. R. (2000) *J. Membr. Biol.* **173,** 165–177.
23. Baumeister, W., Walz, J., Zühl, F. & Seemüller, E. (1998) *Cell* **92,** 367–380.
24. Hayashi, M., Ishibashi, T., Tanaka, K. & Kasahara, M. (1997) *J. Immunol.* **159,** 2760–2770.
25. Takezaki, N., Zaleska-Rutczynska, Z. & Figueroa, F. (2002) *Gene* **282,** 179–187.
26. Abele, R. & Tampé, R. (1999) *Biochim. Biophys. Acta* **1461,** 405–419.
27. Lennon-Duménil, A.-M., Bakker, A. H., Wolf-Bryant, P., Ploegh, H. L. & Lagaudrière-Gesbert, C. (2002) *Curr. Opin. Immunol.* **14,** 15–21.
28. Kanaoka, Y., Ago, H., Inagaki, E., Nanayama, T., Miyano, M., Kikuno, R., Fujii, Y., Eguchi, N., Toh, H., Urade, Y., *et al.* (1997) *Cell* **90,** 1085–1095.
29. Ranes-Goldberg, M. G., Hori, T., Mohan-Peterson, S. & Spits, H. (1993) *J. Immunol.* **151,** 5810–5821.
30. Rees, D. J., Ades, S. E., Singer, S. J. & Hynes, R. O. (1990) *Nature* **347,** 685–689.
31. Schröder, K., Martoglio, B., Hofmann, M., Hölscher, C., Hartmann, E., Prehn, S., Rapoport, T. A. & Dobberstein, B. (1999) *EMBO J.* **18,** 4804–4815.
32. Fichtelius, K. E. (1970) *Lymphology* **1,** 50–59.
33. Klein, J. (1988) in *The Pharmacology of Lymphocytes*, eds. Bray, M. A. & Morley, J. (Springer, Berlin), pp. 11–36.