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John S. Greenspan
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Director

January 24, 1990

Reiko Namikawa, M.D. Ph.D
Research Scientist
SYSTEMIX
3400 West Bayshore Road
Palo Alto, CA 94303

Dear Dr. Namikawa:

I am delighted to learn that our collaborative project on HIV and lymph-nodes is progressing well.

I look forward to discussing further data from the study with you and Dr. McCune.

Thank you for the report.

Sincerely,

A handwritten signature in cursive script, reading "John S. Greenspan".

John S. Greenspan

JSG:ey

cc: Dr. D. Abrams
Ms. Y. De Souza

SYSTEMIX



cc Dr Abrams
Yuan

January 18, 1990

Dr. John S. Greenspan
UCSF
School of Dentistry
Oral AIDS Center
HSW-604
San Francisco, CA 94143-0512

Dear Dr. Greenspan:

Thanks for your assistance in the project to analyze lymph nodes from patients with AIDS. The experiments are ongoing with some interesting results. We analyzed the sections by in situ hybridization with 35S-labelled RNA probes which can hybridize to all species of HIV transcript. After long term exposures(3 weeks) for autoradiography, I found that most of the specimens showed weak signals in the follicular area of the lymph node, probably related to follicular dendritic cells. This has not been published yet as far as I know. It is very interesting should it turn out to be real.

To confirm that this finding is not an artefact, we are going to do the following experiments:

- 1) test the reactivity of other RNA probes for HIV on the same samples (we have some extra slides with which to work),
- 2) test the reactivity of these probes on lymph node sections not infected to HIV.

SYSTEMIX



We will then proceed to direct comparisons between these human nodes and those infected in the SCID-hu mouse.

I look forward to continuing this most interesting collaboration with you and your co-workers.

Sincerely,

A handwritten signature in black ink, appearing to read 'Reiko Namikawa'. The signature is fluid and cursive, with a long, sweeping horizontal stroke at the end.

Reiko Namikawa, M.D. Ph.D
Research Scientist

cc: J.M.McCune



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John S. Greenspan
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January 8, 1990

Dr. Mike McCune
Systemix
3400 West Bayshore Road
Palo Alto, CA 94303

Dear Mike,

I hope you enjoyed your holidays. I will be preparing our annual report during the next two weeks. In it I will be discussing the results of our collaborative work. Have you and Dr. Namikawa found anything interesting in the lymphadenopathy cases we sectioned for you? Have you found any similarities with the SCID-hu mouse lymph nodes and the human lymph nodes?

I would be very interested in hearing about your results. Do not hesitate to contact me if you require further assistance with your project.

Sincerely,

A handwritten signature in black ink, appearing to be "JSG", written over a horizontal line.

John S. Greenspan

JSG:ey

cc: Y. De Souza

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November 21, 1989

Dr. John S. Greenspan
Professor and Chair
School of Dentistry
Department of Stomatology
University of California, San Francisco
S-162, Box 0422
San Francisco, CA 94143

Dear John:

Thank you very much for the slides and the reprints.

By the way, there is ~~some~~ recent data that I have come across since the workshop, regarding some of the topics that we discussed. HIV has been looked for in lymph nodes of patients with lymphadenopathy and HIV infection (reprint enclosed; apparantly this paper has just been indexed in Medline, as I did not see it before the workshop, and I do not read the pathology literature regularly). Apparantly there is also some preliminary data regarding the presence of EBV genomes and antigens in some of these lymph nodes as well, although this has ~~also~~ been published only as part of a symposium's proceedings (which, of course, was on my desk when I returned from Washington); I have not seen this paper in Medline as yet. I have also enclosed this preliminary report, for your interest. I will definately call you the next time I plan on being in San Francisco.

Sincerely,



Ben Z. Katz, M.D.

cc:BZK



NOV 28 1989

Expression of HIV in Lymph Node Cells of LAS Patients

Immunohistology, In Situ Hybridization, and Identification of Target Cells

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Monoclonal antibodies (MAb) anti-HIV core and envelope proteins and *in situ* hybridization, using cDNA HIV probe, were employed to determine which lymph node cells in LAS patients express viral antigens and viral nucleic acids. The results have been correlated with the histologic phases of LAS and with the germinal center lysis detected using DRC-1 MAb directed against follicular dendritic reticulum cells (FDRC). Viral antigens occasionally were detected on high en-

dothelial cells of paracortical venules and frequently on germinal center FDR accessory cells; this last finding correlates well with the extent of FDRC lysis and of CD4+ and CD8+ lymphocyte infiltration in germinal centers. HIV replication, detected by *in situ* hybridization, was observed in mononucleated cells present in T and B areas and, in one case, in flat endothelium. (Am J Pathol 1988, 133:498-506)

IT IS NOW WELL established that the human retrovirus HTLVIII, now renamed human immunodeficiency virus (HIV), is the etiological agent for the lymphadenopathy syndrome (LAS) and for the acquired immune deficiency syndrome (AIDS).^{1,2}

LAS is a generalized and persistent lymphadenitis characterized by several histologic phases; they range from hyperplasia of follicles to follicular involution and ultimately to lymphoid depletion. Quantitative and functional changes of T cell subsets have been described extensively³⁻⁶ during the various phases of LAS. Macrophages and accessory cells, including endothelial cells, play important and specific roles in several immune reactions.⁷⁻¹⁰ The role played by these cells during HIV infection has been investigated in peripheral blood, bone marrow, and lung^{11,12} and in the brain.¹³⁻¹⁵ No studies have been published until now on the role played by accessory and endothelial cells in the pathogenesis of the HIV induced lymph node alterations. We have recently described a marked vascular activation in LAS lymphadenitis¹⁶

and this finding may be related to the cellular mechanisms involved in the pathogenesis of lymph node tissue damages induced by HIV. HIV antigens and DNA-RNA sequences have been described in cells of lymphoid tissues of patients with LAS or AIDS,¹⁶⁻²³ but the nature of the cells infected by HIV and their role in the pathogenetic mechanisms by which the virus induces the various lymph node alterations are still largely unknown. The present study uses *in situ* hybridization with DNA HIV probe and immunohistochemistry to determine which lymph node resident cells express HIV gene products. Viral antigens are expressed by accessory, small lymphoid, and high endo-

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Address reprint requests to Professor Carlo D. Baroni, Second Chair of Pathological Anatomy, Viale Regina Elena 324, 00161 Roma, Italy.

thelial cells; HIV replication takes place in small and large mononucleated cells present in T and B dependent areas and in flat endothelial cells.

Materials and Methods

Tissue Specimens

Axillary and cervical lymph nodes were removed from 50 HIV-positive patients of both sexes (18–39 years old) affected by LAS. They were 34 drug abusers (23 males and 11 females), 7 female partners of drug abusers, 6 male homosexuals, and 3 hemophiliacs. Representative tissue samples from all cases were frozen in liquid nitrogen immediately after surgery. Twenty of these nodes were studied previously with immunohistochemistry^{16,17} and ten with *in situ* hybridization.²²

Controls

Control tissues included 24 lymph nodes obtained from HIV-negative patients not at risk for AIDS: 16 had chronic follicular lymphadenitis, 4 had Hodgkin's disease, and 4 had non-Hodgkin's lymphoma.

Tissue Culture Cell Lines

HIV infected and uninfected H9 cell lines were used as positive and negative controls.

Histology

Tissues samples were fixed in 10% buffered formalin, paraffin embedded, and routinely stained with hematoxylin and eosin (H & E), PAS, Giemsa, and Gomori's for reticulin.

Identification and classification of the various HIV-related histologic changes in lymph nodes were made according to criteria based mainly on follicular alterations. All cases were histologically classified as follows: follicular hyperplasia (FH) without or with follicular fragmentation (FH-FF), follicular involution (FI), and follicular depletion (FD) with or without peculiar vascular lesions similar to the vascular transformation of sinuses. This classification was proposed by a group of pathologists during the Workshop of the European Study Group of HIV-Related Lymphadenopathy, held in Brussels in October 1986.

Immunohistology

Immunostaining was done on 6- μ acetone-fixed cryostat sections. The antibody to HIV antigens in-

Table 1—List of the Antibodies Used

Antibody	Cluster designation	MW (kd)	Main cellular distribution
OKT3*	CD3	19–29	T cells
OKT4*	CD4	55	Helper/inducer T cells, macrophages
OKT8*	CD8	32–33	Cytotoxic/suppressor T cells
DRC-1†	CD35	200	Follicular dendritic reticulum cells
FVIII RA† (antiserum)	NA	–	Endothelial cells
OKIa-1*	NA	29–34	HLA-DR expressing cells
Cytokeratin B*	NA	–	Mature epithelial cells

* Ortho Pharmaceutical, Raritan, NJ.

† Dakopatts, Denmark.

NA, not applicable.

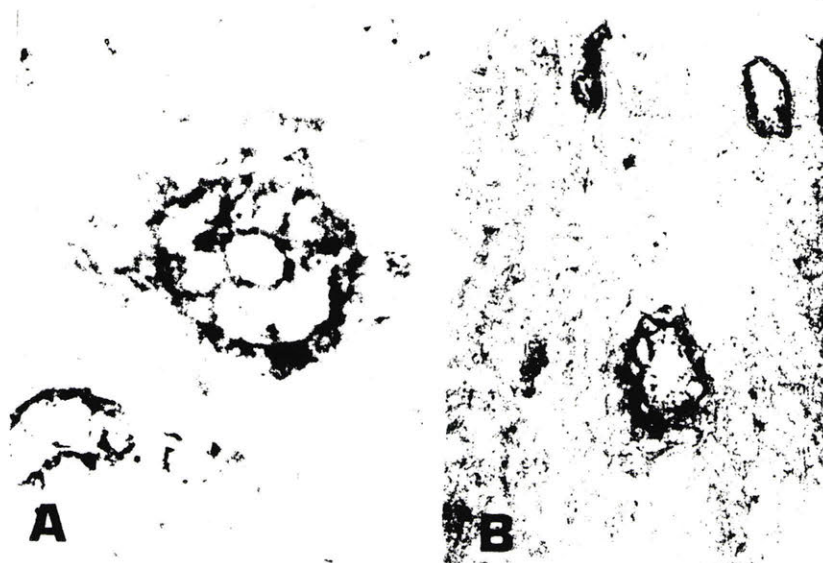
cluded two mouse monoclonal antibodies, M26²⁴ and M25,²⁵ recognizing the HIV major core protein p24 and the HIV envelope protein gp41, respectively. Both monoclonal antibodies were used at the optimal dilution of 1:200 that was established on HIV *in vitro* infected H9 cells.

In addition the sections were incubated with the antibodies listed in Table 1. The sections were then washed for 10 minutes with PBS and reincubated using the indirect avidin-biotin complex method (ABC kit, mouse IgG PK-4002 and rabbit-Vectastain, Vector Laboratories, Burlingame, CA). The reaction product was developed using 0.06% 3-3' diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.03% hydrogen peroxide in PBS for 3–5 minutes; the sections were then rinsed in PBS, stained with hematoxylin and mounted in glycerin.

In Situ Hybridization

The following probes were used: HTLV: SacI, HINDIII fragment, 3' viral HTLV DNA of 5.6 kilobase (kb, encoding LTR, GAG, ENV, LTR sequences) in PBR322 plasmid; HIV: genomic 9Kb SstI-SstI DNA viral insert lambda BH10 inserted into vector SP64;²⁶ HBV: HINDIII whole HBV genome of 3.2 Kb inserted into PBR 322 plasmid; and plasmid: PBR 322 plasmid without any viral insert. *In situ* hybridization was performed on cryostat sections using a nonradioactive labeling of DNA according to a method described recently.²² In brief, an antigenic sulphone group was chemically labeled with the cytosin moieties of the denaturated DNA probe using a commercially available kit (DNA Chemiprobe, Origenics, Israel). Six-micron cryostat sections were quickly fixed with acetone and then covered with 20–30 μ l of the hybridization solution (SSC 2 $\times$, deionized

Figure 1—Immunostaining with the monoclonal antibody OKIa-1 (A). A HEV in the paracortex is intensely stained. (Immunoperoxidase, $\times 1000$) Immunostaining with a monoclonal antibody anti p24 viral core protein (B). Several HEVs in the paracortex are stained. (Immunoperoxidase, $\times 400$)



formamide 45%, dextrane sulfate 10%) containing the labeled probe. After heating to 80 C for 5 minutes to denature tissue DNA,²⁷ hybridization was performed for 18 hours at 37 C in humid chamber. Subsequently the sections were extensively washed for 10 minutes in washing solution (SSC 2 $\times$, Triton 100X 0.05%) at room temperature and incubated 1 hour with a monoclonal antibody anti-sulphone group (DNA Chemiprobe) and with an alkaline phosphatase-conjugated anti-mouse immunoglobulin (DNA Chemiprobe). Finally, the slides were washed with the washing solution, incubated 20 minutes with the chromogenic substrate naphthol AsMx/phosphatase

fast red TR (red granular positivity) or NBT (black granular positivity, DNA Chemiprobe), and mounted in glycerin. Endogenous alkaline phosphatase was inhibited heating the sections at 80 C for 5 minutes.

Restrictive criteria were used to define positive cells to avoid misinterpretations due to background staining. Positivity was considered in those cells showing a high concentration of granules within the cell limit.

Results

HIV-positive patients included 37 men and 13 women, aged 18–39 years. Each had enlarged cervical or axillary lymph nodes, or both.

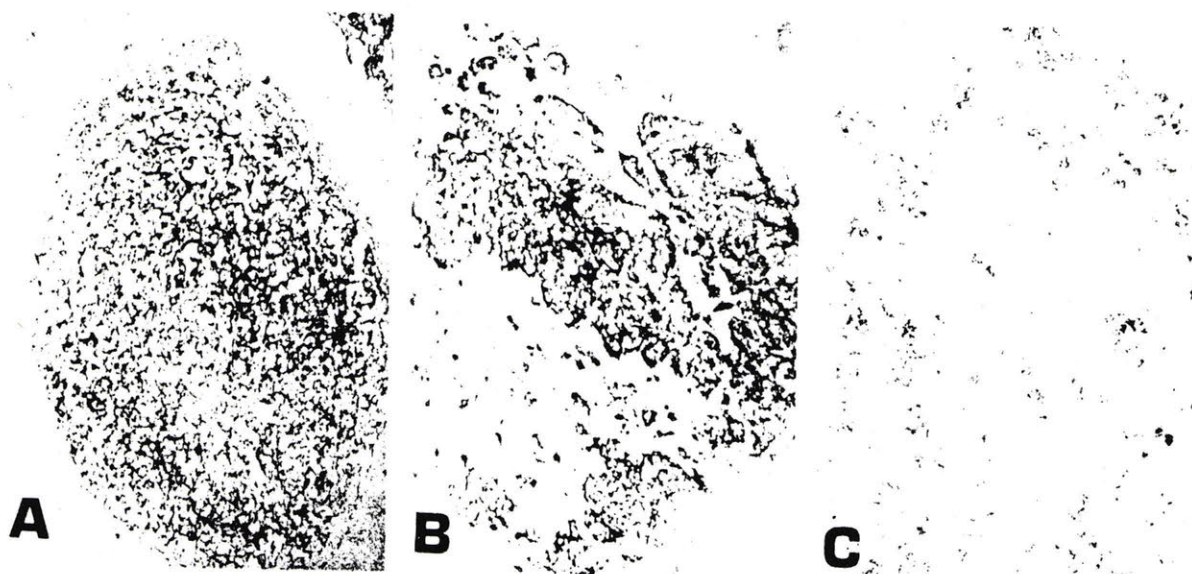


Figure 2—Immunostaining with the monoclonal antibody DRC-1 detecting FDR cells in germinal centers. Note the progressive fragmentation (A, B) and lysis (C) of the DRC-1+ meshwork. (Immunoperoxidase, $\times 100$)

Microscopy

The morphology of the lymph nodes of HIV-positive patients recently presented in the Methods section. The nodes were made in 2–3 cm of FD with maintaining blood vessels constantly of the network. In the nodes (HIV positive), the lining the cells."

Immunohistochemistry

LAS Lymph Nodes

Blood vessel antiserum. T areas. I constantly clonal OKIa-1 cells. (other vessels dendritic displayed stained with of LAS lymph node positive reticular lesions were observed without FDR-1+ infiltration of phocytes (of the DRC-1+ meshwork, the FI, and

By the use of anti-p24 core protein (anti-p24) we have the present lymph nodes obtained tested, all the presence of p24 to that of moreover p24 and DRC-1+ appearance of germinal centers CD8+ lymphocytes positive for

Microscopic Pathology

The major histologic findings observed in lymph nodes of LAS patients have been classified using recently proposed criteria, as specified in Materials and Methods. Briefly a diagnosis of FH without FF was made in 26 cases, of FH with FF in 20, of FI in 2, and of FD with angioimmunoblastic like pattern in the remaining 2 patients. A marked proliferation of small blood vessels in B- and T-dependent areas was constantly observed independently of the histologic pattern. In most cases paracortical high endothelial venules (HEV) were particularly numerous and hyperplastic; the single layer of high endothelium usually lining these venules was replaced by stratified "cubic cells."

Immunohistology

LAS Lymph Nodes

Blood vessels were strongly positive for FVIII RA antiserum, independently of their localization in B or T areas. Endothelial cells of paracortical HEV were constantly strongly immunostained with the monoclonal OK Ia-1, which reacts with HLA-DR expressing cells (Figure 1A), whereas flat endothelial cells of other vessels were completely unreactive. Follicular dendritic reticulum cells (FDR) of germinal centers displayed a regular reticular pattern when immunostained with the antibody DRC-1. In germinal centers of LAS lymph nodes the changes of the DRC-1 positive reticulum paralleled the severity of the histologic lesions well. Three immunohistochemical patterns were observed: 1) normal DRC-1+ reticulum in FH without FF (Figure 2A); 2) focal areas of lysis of the DRC-1+ reticulum paralleling the germinal center infiltration of CD3+/CD4+ and CD3+/CD8+ lymphocytes (Figure 2B);^{3,18,28} and 3) severe to total lysis of the DRC-1+ reticulum that correlates with the FF, the FI, and the cellular depletion (Figure 2C).

By the use of two monoclonal antibodies against p24 core (M26) and gp41 envelope (M25) HIV proteins we have immunohistochemically demonstrated the presence of p24 and gp41 positive cells in lymph nodes obtained from LAS patients. In 30 of 48 cases tested, all characterized by FH, we observed the presence of p24+ cells; they had a reticular pattern similar to that of DRC-1+ FDR (Figures 3A and B); moreover the parallel distribution of cells positive for p24 and DRC-1 MABs is also indicated by the disappearance of the reactivity for both MABs in damaged germinal centers heavily infiltrated by CD4+ and CD8+ lymphocytes. Paracortical HEV were found positive for p24 MABs in four cases (Table 2) charac-

terized by a marked paracortical activation. In two instances numerous HEV were heavily p24 immunostained, thus paralleling the pattern displayed by HLA-DR+ HEV (Figure 1B). In the remaining cases only few p24+ HEV were observed. Furthermore, in five patients few p24+ small and large mono- or binucleated cells, sometimes in perivascular position, were found. It may be noteworthy to say that in one single case cells lining intermediary and medullary sinuses were also found to be p24+. The presence of gp41+ cells was established in four of five patients. In two instances gp41+ cells had a reticular pattern paralleling that of DRC-1+/p24+ FDR. Few gp41+ small and large mononucleated paracortical cells were observed in four patients; in one patient (Table 2) the flat endothelium of a blood vessel was found to be clearly gp41+ (Figure 4).

Controls

HIV-infected H9 cells were stained strongly by M26 and M25 MABs; uninfected H9 cells, neoplastic, and reactive control nodes from HIV negative patients were always unstained. Infected and uninfected H9 cells, LAS, and control nodes were always negative after staining with the nonreactive mouse MAB anti-cytochrome c.

In Situ Hybridization

LAS Lymph Nodes

In situ hybridization, using a Sp64 plasmide containing a 9 Kb HTLVIII viral insert lambda BH10, has been performed in nodes obtained from 19 HIV-positive LAS patients and from 10 control HIV-negative patients not at risk for AIDS. The reaction product was an intracytoplasmic granular staining indicating the expression of viral replication and, in few instances, a nuclear positivity, suggesting the presence of proviral DNA. The staining color was respectively red or black if naphtol/fast red or NBT were used as chromogenic substrate.

In 10 of 19 nodes we observed few small and large mononucleated positive cells (1-20 cells/cryostat section) located in germinal centers and/or in paracortical areas. In one instance a marked cytoplasmic positive *in situ* hybridization was detected in numerous germinal center cells present in a single follicle (Figure 5A). In one case *in situ* hybridization with an HTLVIII probe localized viral nucleic acid in the flat endothelial cells of a blood vessel, thus indicating active viral replication (Figure 6).

Controls

H9-infected cells were strongly positive after *in situ* hybridization with HTLVIII probe (Figure 7A) and

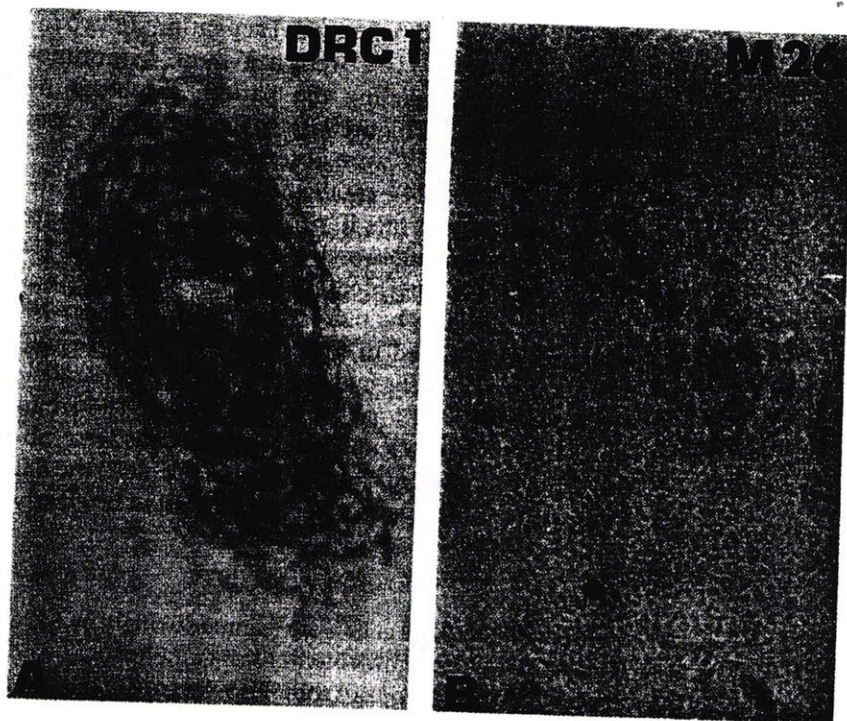


Figure 3—Immunostaining of two consecutive serial sections with the MAbs DRC-1 (A) and M26 (B) detecting respectively FDRG (A) and p24+ cells (B). Note the similar pattern of reticular positivity. (Immunoperoxidase $\times 250$)

Figure 6—Staining of HTLVIII proviral DNA and viral RNA by *in situ* hybridization on a lymph node cryostat section. **A**—Endothelial and mononuclear cells (arrows) are present in a blood vessel (V) and in paracortical area (Px). **B**—Higher magnification of positive endothelial cells. Positivity is indicated by cytoplasmic red granules. (Chromogenic substrate fast red: A $\times 250$; B $\times 1000$)

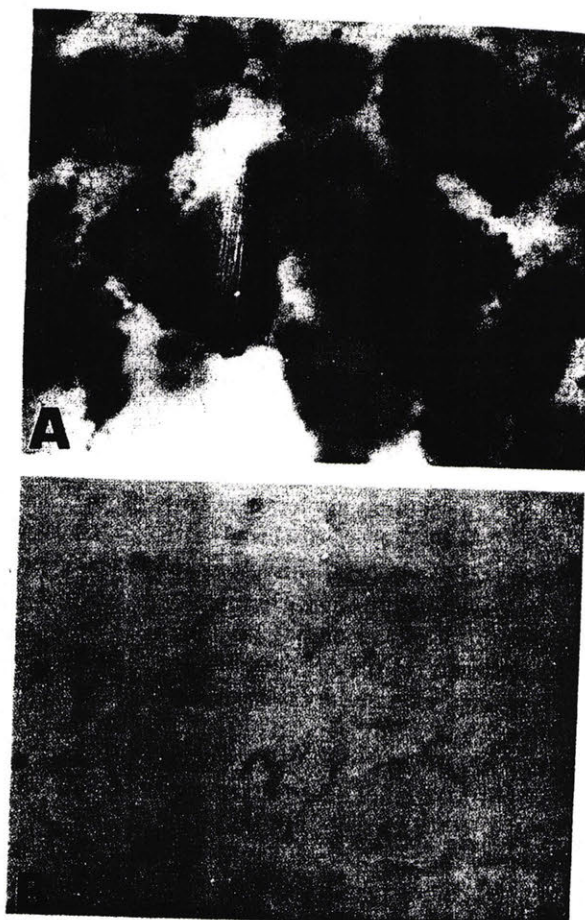
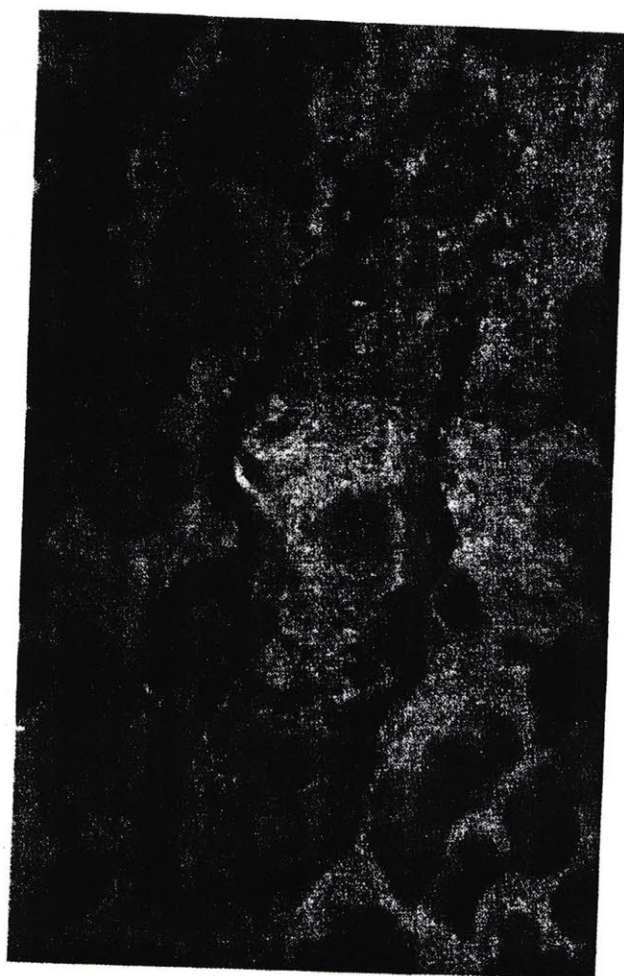


Figure 6
paracortical
Staining
lymph node
cells
HTLV-III p

Figure 6
granules
(Chr)

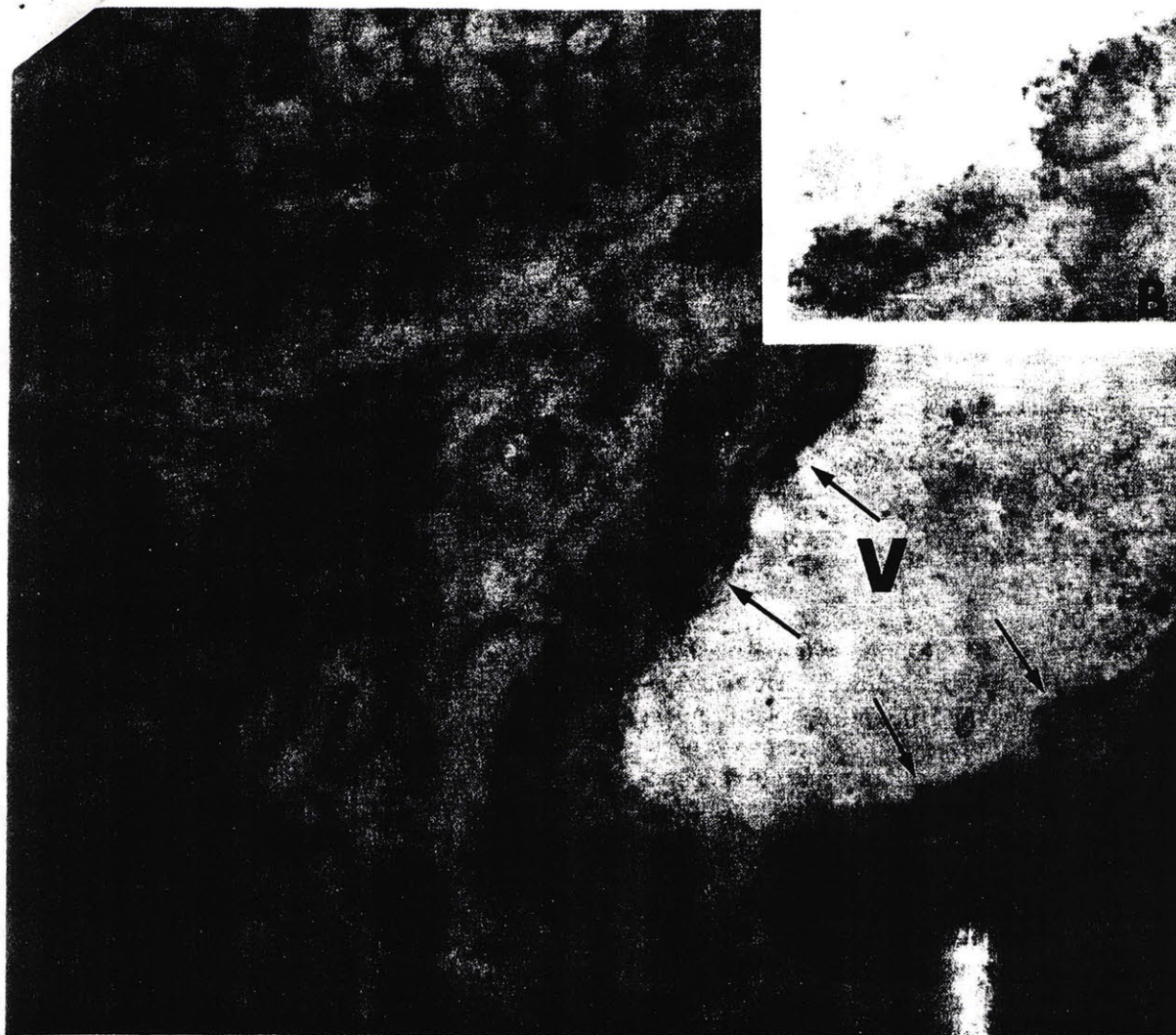


Figure 4—Immunostaining with the MAb M25 detecting gp41+ cells in a paracortical blood vessel. (Immunoperoxidase $\times 1000$), **Figure 5**—Staining of HTLVIII proviral DNA and viral RNA by *in situ* hybridization on a lymph node cryostat section. **A**—Red granules indicate germinal center cells with evident cytoplasmic granular positivity. **B**—Section from an HIV-negative lymph node unstained after *in situ* hybridization with the HTLVIII probe. (Chromogenic substrate fast red $\times 1000$)



Figure 7—H9 line HIV infected (A) and uninfected (B) cells. An evident red granular cytoplasmic staining (A) was observed only in HIV-infected cells. (Chromogenic substrate fast red; $\times 1000$)



Table 2—Results of Immunohistochemistry and *in situ* Hybridization in Lymph Nodes of HIV-positive Patients, in Which Viral Antigens or Genoma Were Found on Blood Vessels

Case	Age/Sex	Histology	Test	Blood vessels			Other lymph node structures		
				PX		GC FEV	PX Mononuclear cells	GC	
				HEV	FEV			Reticular pattern	Mononuclear cells (NOS)
1	41/M	FH	p24	+(rare)	—	—	—	+	—
2	43/M	FH-FH	p24	+(rare)	—	—	+	+	—
3	27/M	FH	p24	+(numerous)	—	—	—	—	—
			genoma	—	—	—	—	—	—
4	23/M	FH	p24	+(numerous)	—	—	—	+	—
			genoma	—	—	—	—	—	—
5	20/M	FH	p24	—	—	—	—	—	+
			genoma	—	+	—	—	—	—
6	29/M	FH	p24	—	—	—	—	—	—
			genoma	—	—	—	—	—	—
			gp41	—	+	—	—	—	—

HEV, high endothelial vessels; FEV, flat endothelial vessels; Px, paracortical areas; GC, germinal centers; NOS, not otherwise specified.

always negative after hybridization with the plasmide only or with the HBV probe. Uninfected H9 cells (Figure 7B), reactive (Figure 5B), and neoplastic nodes from HIV-negative control patients were always negative. LAS lymph nodes hybridized with HTLV-I (7 patients) and HBV (5 patients) probes also were negative.

Discussion

The concept that HIV might have an effect on lymph node accessory and blood vessel cells depends on three groups of observations. First, we have observed that germinal center accessory cells, including follicular dendritic reticulum cells, are concerned with the presence of HIV antigens p24 and gp41. According to our results, p24+ germinal center cells with a reticular pattern were observed in the majority of cases (30 of 48), whereas HIV genome plus germinal center cells were found in only one instance. These findings further support the hypothesis that HIV antigens are trapped as immune complexes in germinal center accessory cells.²⁹⁻³¹ Therefore, we suggest that the development of lymphoid tissue damage could be seen as a sequence of cellular events beginning with the trapping of different viral antigens by competent cells, and subsequently resulting in germinal center lysis with T cell infiltration, and accessory and lymphoid cell damage and death, and ultimately in cellular depletion.

The second group of observations is concerned with the fact that some lymph-node tissue damage in LAS patients may be related to changes of blood vessel cells. Such changes, located mainly in paracortical HEV, could be demonstrated microscopically and im-

munohistologically independently of the histologic phase of the disease.

The third group of data are those demonstrating viral antigens and viral replication in endothelial cells. We believe that viral antigens could be one of the factors responsible for the marked blood vessel activation; furthermore endothelial cells, in which viral replication may take place, might represent a virus reservoir from which the infection can spread and become detrimental to other cells. These findings suggest that endothelial cells also play a role in the pathogenesis of HIV-induced lymph node lesions. It seems, therefore, that the term "vascular change," as applied to the pathogenesis of HIV virus-related lymphoid tissue injuries, includes several phenomena. Two different types of lymph node paracortical blood vessels appear to be involved in HIV infection: the paracortical HEV characterized by marked HLA-DR positivity and by a possible role in antigen presentation,^{9,10} and the flat endothelial vessels. We have reported previously^{16,17} that in LAS patients p24 HIV core antigen is observed in activated paracortical HEV. The present study confirms this observation using a larger LAS patient population, but we have never observed gp41 envelope antigen and replicating virus in HEV. The presence in these patients of p24 core antigen and the simultaneous absence of gp41 envelope antigen and of viral genome in HLA-DR activated HEV suggest that these peculiar endothelial cells are not HIV infected. Nevertheless they may play a possible accessorylike role in HIV antigen presentation, as has been already suggested for macrophages.¹¹ This interpretation is also supported by our observation, recently confirmed by Ward et al,¹⁹ that p24 positivity could be detected in high endothelial cells during the initial phases of LAS, when the lymph node activation starts.

It was described previously that, in lymph nodes, HIV replication is present in rare lymphoid cells;^{20,22,23} the present study confirms that the number of cells containing HIV genome is usually limited. It has been recently reported that lentivirus infection is characterized, at tissue level, by very low numbers of cells positive for viral RNA.³² The low numbers of HIV genome positive cells could likewise be explained on the basis of the sequence homology existing between HIV and lentivirus.^{33,34} In a single case the *in situ* hybridization has proven the presence of HIV genome in HLA-DR negative flat endothelial cells of a vessel, thus suggesting that HIV is able to infect cells other than macrophages¹¹⁻¹³ and CD4+ lymphocytes.³⁵ Our data are in agreement with the recent observation of HIV replication in brain capillary endothelial cells of AIDS patients.¹⁴ The demonstration of gp41 envelope antigen in flat endothelial cells of a LAS lymph node (Figure 4) further support this interpretation.

In the present and in previous investigations^{3,16} we were unable to demonstrate CD4 antigen on endothelial cells. Therefore, it could be possible that surface antigens other than CD4 may be involved in the cellular mechanisms of HIV recognition and infection. It has been reported that, besides CD4+ lymphocytes, HTLV I also infects human endothelial cells *in vitro*,³⁶ thus inducing the appearance of FVIII RA+ and HLA-DR- giant cells.³⁷ This finding agrees with our observation that HIV infection also may occur at tissue level in HLA-DR negative endothelial cells.

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basically an earlier version of this paper

11

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Acknowledgment

The authors thank Dr. Robert C. Gallo for providing monoclonal antibodies anti p24 and anti gp41 and the HTLVIII probe.

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carcinogenesis. Further studies are in progress to see if this particular plant substance induces chromosome translocations, such as that between No.8 and No.14 chromosomes characteristic to Burkitt's lymphoma and responsible for c-myc proto-oncogene activation(8).

ACKNOWLEDGEMENTS

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Humana, NJ, 1988

EPSTEIN-BARR VIRUS ANTIGENS AND GENOME IN LYMPH NODES FROM PATIENTS WITH PERSISTENT GENERALIZED LYMPHADENOPATHY (PGL).

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The HIV associated persistent generalized lymphadenopathy (PGL) is characterized by enlarged lymph nodes with prominent activation of B germinal centers (1). Some patients affected by PGL develop B cell lymphomas, thus suggesting that PGL may be a predisposing condition. EBV infects and immortalizes B lymphocytes; it has been suggested that EBV may be associated with the development of B lymphomas in immunocompromised hosts. EBV-associated B lymphomas have been observed in patients with primary immunodeficiency (2); furthermore B lymphomas with integrated EBV genome have been described in patients receiving immunosuppressive therapy because of organ transplantation (3). AIDS-lymphomas show numerous analogies with EBV-lymphomas: they are B lymphoblastic and express similar chromosomal translocations and c-myc rearrangements (4,5). Furthermore EBV sequences were found integrated in the DNA of leukemia cells of AIDS patients (6).

In this study we have investigated the presence, at tissue level, of EBV (Table 1) using immunohistology on cryostat sections of 50 nodes with PGL. The following monoclonal antibodies (MoAbs) were employed: anti HIV p24 major core protein (7), anti EBV-VCA (8) and anti EBV-EA (9). The immune reaction product was revealed using an avidin-biotin peroxidase complex. EBV genome was

Table 1

Patients	Serology		LN° Antigens		LN Genome	
	HIV	EBV	HIV	EBV	HIV	EBV
50 (32M+18F)	50/50*	42/50	43/50	2/50	+/-°	0/30

* Number of positive patients/total number of patients

° Only few cells in the Lymph Nodes (LN) are HIV genome positive (11).

investigated by in situ hybridization on cryostat sections of 30 lymph nodes, which were hybridized with a biotinylated EBV-DNA probe (Bioprobe TM, Enzo Biochem, NY, USA); the reaction was enzymatically detected with an avidin-biotin peroxidase complex. Furthermore EBV genome was studied by Southern blot hybridization of DNA extracted from 7 frozen lymph nodes with an EBV cloned probe (EBV Bam HI-W fragment, about 3.07 Kb long), nick translated with ³²P labeled deoxycytidine 5' triphosphate.

Immunoreactivity for HIV p24 protein was detected in 43 out of 50 nodes obtained from HIV+ patients; the positivity was confined to the germinal centers exhibiting a reticular pattern, similar to that of the follicular dendritic reticulum cells.

Although the majority of the patients (42/50) had significant anti-EBV titers, the presence of EBV antigens and genome was rarely detected in the lymph nodes. Actually EA and VCA could be observed only in 2 out of 50 lymph nodes, in a few centrocyte-like cells mainly located in germinal centers. The presence of EBV genome was investigated by in situ hybridization and by Southern blot analysis; interestingly enough the EBV genome could not be demonstrated at tissue level.

Cell lines were used as positive controls for the demonstration of EBV antigens and genome (Table 2): 20-30% of the B95-8 cells were immunostained with anti EBV-VCA and EBV-EA MoAbs. Furthermore, 15-30% of the B95-8 cells showed a nuclear positivity following EBV-DNA in situ

Table 2

Controls	Antigens			In situ	
	HIV	EBV-EA	EBV-VCA	HIV	EBV
HIV infected H9	45*	-	-	+	-
EBV-producer B95-8	-	20-30	20-30	-	15-30
P3HR-1 infected Raji	-	5	-	-	+
EBV-non producer Raji	-	-	-	-	+
K562	-	-	-	-	-
Carcinoma EBV-DNA+	nd**	nd	nd	nd	+++
Carcinoma EBV-DNA-	nd	nd	nd	nd	-

* Percentage of positive cells.

** Paraffin embedded tissue (viral Ags undetectable).

hybridization. Approximately 5% of P3HR-1 infected Raji cells expressed EBV-EA; on the contrary EBV-non producer Raji cells and K562 cells were constantly negative for EBV antigens. However, both non infected Raji and P3HR-1 infected Raji cells showed integrated EBV-DNA. The EBV-DNA probe was also used to hybridize the cells of two nasopharyngeal carcinomas. In one case, in which EBV was integrated (10), numerous neoplastic cells were EBV genome positive.

Our results confirm previous reports showing that HIV antigens are present in germinal centers of the majority of lymph nodes involved by PGL (11), which is characterized by prominent activation of the B dependent areas and by polyclonal hypergammaglobulinemia. The stimulus responsible for B cell activation is not yet well known. It has been suggested that HIV antigens might stimulate germinal center B lymphocytes through a continuous presentation of HIV-immunocomplexes (11). However it has been also proposed that EBV, perhaps reactivated by HIV infection, could be responsible for the B cell polyclonal activation (12).

Our results show that only rare EBV-VCA and EBV-EA positive cells can be detected in few lymph nodes from HIV+ patients; furthermore, EBV-genome positive cells were never observed. The absence of EBV-antigens and genome, at lymphoid tissue level, might suggest that EBV is not directly involved in the development of PGL. However, a silent EBV infection, with few infected cells and/or with few viral copies per cell, cannot be completely ruled out.

Acknowledgements. Prof.G.Pearson kindly provided anti EBV-VCA and EBV-EA MoAbs and Dr.R.C.Gallo anti HIV-p24 MoAb. We thank Mr.F.Marziali for his excellent technical assistance. Supported by the Progetto "AIDS" Ministero della Sanita' and by Associazione Italiana Ricerca sul Cancro.

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INTERACTION OF HBLV (HHV-6) WITH EBV AND HIV

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INTRODUCTION

A novel human herpesvirus, HBLV, also known as human herpesvirus-6, was originally isolated by us from AIDS and from patients with a variety of hematological disorders (1,2). Since then, HHV-6 has been isolated from AIDS patients from Africa, healthy blood donors and from infants with roseola infantum (*Exanthem subitum*) (3,4,5,6) by others and from patients with chronic fatigue syndrome by us (7). HHV-6 is a new member of the human herpesvirus family, and is different from other herpesviruses by immunologic and molecular analysis (1,2,8). However, the virus showed restricted sequence similarity to human cyto-megalovirus (9) and Marek's disease herpesvirus of chickens by DNA hybridization (10). HHV-6 possesses wide cell tropism and replicates and lyses fresh and established human CD₄ positive cells (7,11,12). Since HHV-6 was isolated from HIV-1 positive AIDS patients and HHV-6 DNA was found in EBV genome positive African Burkitt's lymphoma (13), we investigated the interaction of HHV-6 with EBV and HIV-1.

9 Oct 89

John,

I saw Reiko & Mike ~~to~~ today. They selected 15 cases for RNA analysis. They brought over premeasured chemicals, vials, coated slides, & protocols.

I have enclosed a copy of their request sheet. The sections should be cut on a disposable knife to prevent RNA / RNAase contamination.

Yvonne

OK.
Do Ind Tol Blw,

SYSTEMIX



August 30, 1989

John S. Greenspan, MD
Oral AIDS Center
HSW-604
UCSF
San Francisco, CA 94143

Dear John:

Thanks for your letter. We are all eager to start.

After looking over the list, Reiko will contact you or Yvonne to go over the details of collecting the cryosections. Perhaps she can just go up there one day with slides, be there while they're cut, and then return here promptly to begin the hybridizations.

We look forward to seeing this data!

Talk to you soon.

Yours sincerely,

A handwritten signature in dark ink, appearing to read 'J. M. Mc Cune'.

J. M. Mc Cune, MD, PhD
Scientific Director

JMM:dsp

cc: Reiko Namikawa, MD, PhD
Ms. Yvonne De Souza
Donald Abrams, MD



University of California
School of Dentistry
Oral AIDS Center
HSW-604
San Francisco, California 94143-0512
(415) 476-9169

August 29, 1989

J. M. Mc Cune, M.D., PhD
Scientific Director
SYSTEMIX
3400 West Bayshore Road
Palo Alto, CA 94303

Dear Mike,

Thank you very much for inviting me and Yvonne De Souza to see your facilities and to discuss the specimen bank as well as our research projects. We were impressed with the fact that you have developed a mouse model that has an immune system similar to that of the human. We appreciate the importance of validating the similarities between human and SCID-hu mouse HIV infection.

I have talked to Don Abrams about your research interests. He is willing to have you use some of his cases to compare with the pathology exhibited in the nodes of the SCID-hu mouse nodes.

Attached to this letter is a printout of the lymph nodes currently present in our specimen bank. You will see that several are probably of interest. We will be more than willing to cryosection some of the nodes for you and place sections on the coated slides you will send us.

If you have any questions please do not hesitate to contact me at 476-2220.

Sincerely,

A handwritten signature in black ink, appearing to read "JSG".

John S. Greenspan

JSG:ey
attachment

cc: Dr. Donald Abrams
Ms. Yvonne De Souza

Record#	TISSUENUM	PATHNUM	DXDATE	DATEREC	DX	TISSUE	SOURCE	COMMENTS
1	R82-097		04/23/82	06/16/82	AIDS	Node	Drew	
2	R82-098		01/19/82	06/16/82	AIDS	Node	Drew	
3	R82-099		04/14/82	06/16/82	AIDS	Node	Drew	
4	R82-101		03/30/82	06/16/82	AIDS	Node	Drew	
5	R82-102		04/14/82	06/16/82	AIDS	Node	Drew	
6	R82-134	SP82-5428	07/06/82	07/06/82	KS	Node	Abrams	
7	R82-152		07/12/82	07/12/82	AIDS	Node	Abrams	
8	R82-153	SP82-5673	07/13/82	07/13/82	KS	Node	Conant	
9	R82-163		07/21/83	07/21/82	LNS	Node	Abrams	
10	R82-164		07/22/82	07/22/82	AIDS	Node	Deckstead	
11	R82-190		08/19/82	07/19/82	AIDS	Node	Abrams	
12	R82-200	SP82-26925	08/26/82	08/26/82	KS	Node	Conant	
13	R82-201	SP82-7017	08/26/82	08/26/82	KS	Node	Conant	
14	R82-210		09/22/82	09/22/82	AIDS	Node	Abrams	
15	R82-215	SP82-8004	09/30/82	09/30/82	KS	Node	Conant	
16	R82-224		10/26/82	10/26/82	AIDS	Node	Abrams	
17	R82-237	SP82-8954	11/04/82	11/04/82	KS	Node	Deckstead	
18	R82-240	SP82-9496	/ /	11/18/82	KS	Node	Conant	
19	R82-241	SP82-9475	/ /	11/18/82	KS	Node	Conant	
20	R82-244		11/30/82	11/30/82	AIDS	Node	Abrams	
21	R82-245	SP82-9753	11/30/82	11/30/82	KS	Node	Conant	
22	R82-250		12/09/82	12/09/82	AIDS	Node	Abrams	
23	R82-251	Sp82-10278	12/16/82	12/16/82	KS	Node	Conant	
24	R83-007	SP83-263	01/11/83	01/11/83	KS	Node	Conant	
25	R83-013	SP82-8760	/ /	01/12/83	AIDS	Node	Deckstead	
26	R83-015	SP81-7819	/ /	10/18/83	KS	Node	Conant	
27	R83-021	SP83-575	01/20/83	01/20/83	KS	Node	Conant	
28	R83-026		02/10/83	02/10/83	AIDS	Node	Abrams	
29	R83-045	SP93-1611	02/24/83	02/24/83	KS	Node	Conant	
30	R83-046	SP83-1611	02/24/83	02/24/83	KS	Node	Conant	
31	R83-071	SP83-2462	03/24/83	03/29/83	AIDS	Node	Deckstead	

32	R83-074	SP83-2664	03/31/83 03/31/83 KS	Node	Conant	
33	R83-093		05/03/83 05/03/83 KS	Node	Levy/Ziegler	
34	R83-112A	SP83-4943	06/20/83 06/20/83 LNS	Node	Deckstead	2 pieces .5cm x .5cm
35	R83-129		07/06/83 07/13/83 KS,LNS,OI	Node	Ziegler	Autopsy.lymphoma.None left.
36	R83-155	SP83-5822	07/15/83 07/15/83 KS	Node	Katzen	Reactive
37	R83-156	SP83-5822	07/15/83 07/15/83 KS	Node	Katzen	Lymphoma on H&E.
38	R83-218	SP83-11315	11/30/83 11/30/83 LNS	Node	Deckstead	2 pieces 1cm x .5,.8cm
39	R84-007	PT2611267	01/13/84 01/13/84 LNS	Node	Conant	2 pieces .5cm x .5cm
40	R84-018C	558647441	02/08/84 02/08/84 KS,HSV	Node-normal	Ziegler	Autopsy. 2 pieces gone, 1 left.
41	R84-043	SP84-4332	04/20/84 04/20/84 AIDS?	Node	Deckstead	History of LNS.
42	R84-098		07/09/84 07/09/84 R/O AIDS	Node	Hohn	
43	R84-117		07/30/84 07/30/84 AIDS,CMV	Node	Adams	Autopsy
44	R84-123		08/02/84 08/02/84 KS	Node	Volberding	Autopsy
45	R84-124	990002223850	08/08/84 08/08/84 AIDS?	Node	Levy/Children H	
46	R84-137		09/07/84 09/07/84 LNS	Node	Volberding	
47	R84-184		10/25/84 10/25/84 T-cell lymph	Node	Mess	
48	R85-014		01/29/85 01/29/85	Node	SFGH	
49	R85-047		03/28/85 03/28/85 AIDS?	Node	Deckstead	
50	R85-072	SP85-4880	04/30/85 04/30/85 PCP.LNS	Node-2	Deckstead	

SYSTEMIX



April 21, 1989

Dr. John Greenspan
AIDS Specimen Bank
Oral AIDS Center
HSW 604
UCSF-Moffitt
San Francisco, Ca. 94143-0512

Dear Dr. Greenspan:

I was made aware of the Specimen Bank through the Research Resources Reporter (2/89) and have just spoken with Yvonne DeSousa by phone. She suggested that I contact you directly.

I am presently the Scientific Director of a start-up biotech company in Palo Alto. "SyStemix" was founded by myself, Irv Weissman, David Baltimore, and Lee Hood to explore a number of research directions in the SCID-hu mouse. This mouse model, developed at Stanford, was initially designed to evaluate the pathophysiology of HIV infection in vivo, in the setting of a small animal with interactive human hematolymphoid organs. We think now that it may also be useful in testing anti-virals against HIV (as well as a number of other viruses which cause disease only in man), in the isolation of human hematopoietic precursor cells, and in the derivation of functional human T and B cells.

I enclose two reprints. The first (Science, 241: 1632, 1988) describes the model system. The second (Science, 242: 1684, 1988) presents evidence that the system is permissive for infection with HIV. One of the most informative assays that we have used has been simultaneous in situ hybridization/immunohistochemistry on paraformaldehyde-fixed tissue (see Fig. 2 of the 12/23 paper). The tissues that have been analyzed to date in this fashion are those that we have actually implanted into the mice (and then infected). We would find it of great interest to compare the results which are obtained in this system to the histopathology of nodes (or other hematolymphoid organs) taken directly from HIV-infected patients.

SYSTEMIX



Dr. John Greenspan
April 21, 1989
Page 2

For that purpose, of course, we need tissue. I was a medical resident at UCSF starting in 1982 and remember that lymph node biopsies were not a rare event back then. Now, still working at Ward 86, it's clear that things have changed. As a start in this comparison study, any frozen tissue would be worth studying. This would be particularly interesting if there were clinical information about the donor in tandem. The tissue would best be from organs such as lymph node, thymus, spleen, gut, or skin. It is optimally fixed first in 4% paraformaldehyde and then frozen, but flash frozen tissue is perfectly suitable (we have a cryostat committed to Biosafety Level 3 work). The tissue sections will be stained with a number of polyclonal and monoclonal antibodies against HIV structural proteins or against phenotypic markers of cell subpopulations. They will then be subjected to in situ hybridization using a number of subgenomic RNA probes from the HIV genome. The protocols are well established here and are described (or referenced) within the enclosed reprints.

I'm not sure how you normally handle requests such as this. Any arrangement would be fine by us. Perhaps most fun would be to do the study as a collaboration (this would be particularly the case if it were possible to correlate the information gained from histopathology with clinical data). Let me know.

In addition to the reprints, I enclose (at Yvonne's suggestion) my CV and a simple brochure describing the company.

I look forward to hearing from you soon.

Yours sincerely,

J. M. Mc Cune, MD, PhD
Scientific Director

cc: Reiko Namikawa, MD, PhD
Barbara Lee

MEMORANDUM OF CALL

TO:

JSG

☐ YOU WERE CALLED BY— ☐ YOU WERE VISITED BY—

OF (Organization)

Date and

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☐ WILL CALL AGAIN

☐ IS WAITING TO SEE YOU

☐ RETURNED YOUR CALL

☐ WISHES AN APPOINTMENT

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MESSAGE

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Aug 17 9:30 for meeting

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L. Nades

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- ② " " its like
- ③ Talk to Don Abrams

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MEMORANDUM OF CALL

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☐ YOU WERE CALLED BY— ☐ YOU WERE VISITED BY—

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MESSAGE

Mike McCune picked
Aug 17 9:30 for meeting

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CSN

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7-10

TIME

CURRICULUM VITAE

Joseph Mc Crary (Mike) Mc Cune III

Date and Place of Birth

[REDACTED]
[REDACTED]

Home Address

[REDACTED]
[REDACTED] [REDACTED]

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1984-1986

Infectious disease fellow
University of California, San Francisco, CA

1984-1987

Physician, General Medical Clinic
San Francisco General Hospital

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Physician, AIDS Clinic
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POSTDOCTORAL TRAINING

1985-1988

Postdoctoral fellow
Department of Pathology
Stanford University School of Medicine
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1988-present

Scientific Director and Founder
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AWARDS AND HONORS

1972-1975

National Merit Scholarship

1972-1975

Harvard National Scholarship

1973

NIH Summer Research Fellowship
Lab: Dr. Georg Springer, Northwestern
University, Evanston, IL.

1973-1975

Henry and Camille Dreyfus Scholarship
Lab: Dr. Jack Strominger
Harvard College, Cambridge, MA

1975-1976

Michael C. Rockefeller Memorial Fellowship
(Harvard Travelling Fellow: study of
traditional medical systems in Botswana
and Sri Lanka)

1986-1989

Pfizer Postdoctoral Fellowship
Infectious Diseases

1987

Diplomate, Internal Medicine

RESEARCH EXPERIENCE

1973

Immunochemistry
Dr. Georg Springer
Northwestern University, Evanston, IL

1973-1975
histocompatibility

Immunochemistry of major
antigens
Dr. Jack Strominger
Harvard College, Cambridge, MA

1976-1982

Immunology/Cell Biology
Dr. Henry Kunkel/Dr. Gunter Blobel
The Rockefeller University, New York, NY

1984-1988

Retroviral pathogenesis
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Stanford University School of Medicine
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TEACHING EXPERIENCE

1973-1975

Lab instructor: inorganic chemistry
Harvard College, Cambridge, MA

1981

Immunochemistry and Cellular Immunology
Course, Rockefeller University (directors:
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Cell Biology Course, Rockefeller University
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1982-1984

Residency training program
University of California, San Francisco

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HIV INFECTION OF THE SCID-hu MOUSE

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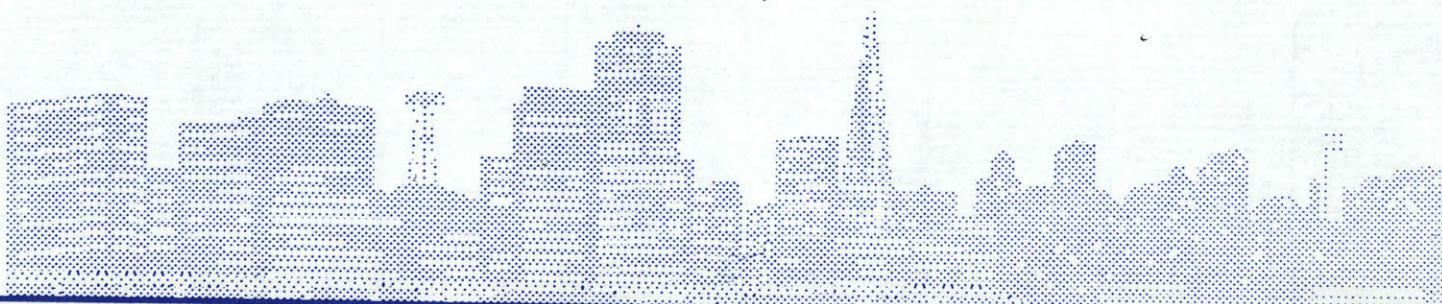
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SCIENTIFIC DIRECTOR
SySTEMICS, INC.

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**The SCID-hu Mouse: Murine Model for the
Analysis of Human Hematolymphoid
Differentiation and Function**

J. M. McCUNE, R. NAMIKAWA, H. KANESHIMA, L. D. SHULTZ,
M. LIEBERMAN, AND I. L. WEISSMAN

The SCID-hu Mouse: Murine Model for the Analysis of Human Hematolymphoid Differentiation and Function

J. M. McCUNE, R. NAMIKAWA, H. KANESHIMA, L. D. SHULTZ,
M. LIEBERMAN, I. L. WEISSMAN

The study of human hematopoietic cells and the human immune system is hampered by the lack of a suitable experimental model. Experimental data are presented showing that human fetal liver hematopoietic cells, human fetal thymus, and human fetal lymph node support the differentiation of mature human T cells and B cells after engraftment into mice with genetically determined severe combined immunodeficiency. The resultant SCID-hu mice are found to have a transient wave of human CD4⁺ and CD8⁺ T cells and human IgG (immunoglobulin G) in the peripheral circulation. The functional status of the human immune system within this mouse model is not yet known.

TO APPROXIMATE THE EVALUATION OF DISEASE STATES IN man, biomedical research has relied heavily upon animal models. Of these, experiments with the laboratory mouse have contributed much to our understanding of the immune system, the cells involved, the products that they express, and their differentiation pathways. After immunization *in vivo*, murine splenic B cells can be immortalized as hybridoma lines making monoclonal antibodies (1), and functional, antigen-reactive murine T cells can easily be cloned (2). After adoptive transfer into lethally irradiated hosts, the murine pluripotent hematopoietic stem cell can be identified, purified, and studied (3, 4). These are important findings. They are not, however, easily or directly applicable to man; except in rare

circumstances, humans cannot be subjected to experimental immunizations or to lethal irradiation and could not provide internal lymphoid organs.

The emergence of the acquired immunodeficiency syndrome (AIDS) underscores the need for direct and comprehensive analysis of the human immune system (5). With this epidemic, as well as with those associated with other human lymphotropic retroviruses, little is known about the course of infection *in vivo*. In the absence of a testable model, the accumulation of knowledge may be slow. Clinical trials represent the only available means of evaluating therapeutic or prophylactic modalities. Although similar animal retroviruses are associated with immunodeficiency states, in no case is the virus identical to human immunodeficiency virus or the disease identical to AIDS. Indeed, if any pertinent knowledge has been gained from the study of animal retroviruses, it is that retroviruses affecting man are best (and perhaps only) studied in the context of human, and not animal cells.

We have taken an alternative approach, one that might create an animal model precisely for the study of the human immune system, its physiology, and its pathophysiology. We now present a method by which the human T and B cell lineages and their hematopoietic precursors can be obtained, transplanted, and observed to differentiate within a mouse.

Several key concepts of immunology guide these experiments.

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First, during fetal life, tolerance to "self" is governed not only by genetic constraints, but also by the environment in which the developing immune system resides. In the experiments of Billingham, Brent, and Medawar (6), the introduction of A strain tissue into the developing embryos of CBA mice permitted the resultant offspring to accept A as well as CBA strain skin grafts. Second, self-nonself education of T cells occurs primarily in the thymus. Thus, the development of tolerance and major histocompatibility complex (MHC) restriction in the T cell lineage is dictated primarily by the thymic microenvironment in which T stem cells initially differentiate (7, 8). Finally, after T cells are mature, self-recognition is fixed; MHC-disparate cells are rejected.

We reasoned that human T cell differentiation might proceed in a physiologic fashion, provided that human hematopoietic stem cells and human thymus were introduced together into a mouse which itself was unable to reject them. We therefore sought to use a mouse stock that expresses a specific defect in lymphocyte maturation so that the absence of murine T and B cells might facilitate the engraftment of human hematopoietic and lymphoid tissues.

C.B-17 *scid/scid* mice (congenic partners of BALB/cAn) lack functional T cells and B cells (9–11). Homozygotes (hereafter designated as SCID mice) have severe combined immunodeficiency, an inability to mount an effective cellular or humoral immune response to foreign antigens. Usually, these mice die of opportunistic infection at an early age; often, the causative agent is *Pneumocystis carinii*. It is thought that the inherent deficiency is related to a defective recombinase function; that is, antigen receptor genes in differentiating T and B cells are not productively rearranged (12). SCID mice are otherwise replete with all other hematopoietic lineages, and in fact show a relative increase in the granulocytic series (13, 14). Their hematopoietic microenvironment, including the thymic stroma, is intact. When mice are engrafted with syngeneic bone marrow cells, reconstitution with functional T cells and B cells can occur. The absence of bone marrow engraftment results in acceptance of MHC-disparate allogeneic skin grafts. Indeed, xenogeneic hybridoma cells grow well without rejection when placed intraperitoneally within these mice (15).

In order to reconstitute SCID mice with human T cells, a source of human hematopoietic progenitors of thymocytes is required. From studies in murine and human systems, it is clear that such cells may be found in adult bone marrow. Careful subfractionation yielded 0.05 percent of murine bone marrow cells that represented a source of pluripotent hematopoietic stem cells. When these cells are given intravenously, 20 to 30 of them can reconstitute 50 percent of lethally irradiated mice (4). In humans, donor bone marrow depleted of mature T cells may be used to reconstitute immunodeficient patients; in the absence of such depletion, the donor T cells are responsible for a graft-versus-host immune reaction. An alternative source of hematopoietic stem cells is the human fetal liver. This organ, the major site of hematopoiesis in humans between 8 to 24 weeks of gestation, contains progenitors for all hematolymphoid progenitors, including the erythroid, the myelomonocytic, and the lymphoid lineages (16). Most, if not all, of the fetal liver T cell progenitors have not yet migrated to and through the human fetal thymus. Accordingly, they are not committed to a given pattern of self-nonself recognition and have proved to be a reliable source of cells for engraftment, even without the preliminary elimination of mature T cells (17).

Here, we describe the hematochimeric SCID-hu mouse, formed by the engraftment of human fetal liver, thymus, and lymph node into the SCID mouse, in such a manner that human stem cell differentiation may be observed to occur.

Construction of the SCID-hu mouse. Human fetal thymus, liver, lymph node, and spleen were obtained and prepared for

introduction into SCID mice by surgical implantation or by intravenous injection (see legend to Fig. 1) (18). The resultant mice were identified by code, for example, X/Y/Z, wherein X refers to the donor code of the thymus implant, Y to the donor code of the fetal liver implant, and Z to the donor code of the fetal lymph node (or spleen) implant. The donor codes, in turn, were catalogued with information related to the gestational age of the fetus and the results of typing for common HLA class I antigens. With a bank of frozen tissue available, it becomes readily possible to construct SCID-hu mice with a variety of human lymphoid organs of defined (and, as desired, different) genetic origin.

In an initial experiment with 21 SCID mice, 10 were segregated to an untreated control group. During the next weeks, these animals showed signs of illness, including weight loss, ruffled fur, and a rapid respiratory rate. All were dead within 4 months. Such a syndrome in SCID mice is most often attributable to opportunistic infection, including that caused by *Pneumocystis carinii*. We subsequently observed that SCID colonies can be easily maintained on prophylactic antibiotics with trimethoprim-sulfamethoxazole (TMS) (see legend to Fig. 1) (19–21). No further outbreaks of disease have occurred; all mice now have the appearance of a healthy BALB/c mouse.

Eleven other SCID mice in this experiment were converted into SCID-hu mice; they were implanted with human fetal thymus and then given an intravenous or intrathymic injection of human fetal liver cells. Even in the absence of TMS prophylaxis, all of these mice remained healthy. Among this group, some were specifically killed for analysis; others are still alive 17 months later.

A reconstituted human immune system may have protected the SCID-hu mice against the acquisition of opportunistic infection. While exploring this possibility, we made the following observations.

Human fetal thymus growth and microscopic anatomy. The human fetal thymus undergoes considerable growth when implanted under the kidney capsule of a SCID mouse. A thymic graft (10 by 5 by 5 mm) is shown in Fig. 1; 13 weeks earlier, this tissue had been implanted as a thymic lobe (0.5 by 0.5 by 2 mm) from a donor in the ninth gestational week. Vasculature, of yet undetermined origin, is clearly evident. Our experience (with approximately 200 thymic implants of this type) indicates that growth potential is at least partially related to the gestational age of the donor, and optimal if used before the 20th gestational week.

The microscopic anatomy of the SCID-hu thymus can bear close resemblance to that of its normal human counterpart (22). The cortical and medullary compartments are well demarcated by immunohistochemical stains for the epithelial antigens CDR2 and MD1 (23) (Fig. 2, A and B). The cortex, but not the medulla, is densely populated with CD1⁺ thymocytes (Fig. 2C); reciprocally, medullary cells are readily stained with antibodies to MHC class I antigens (as shown below). Immunohistochemical stains for human CD4 and CD8 reveal reactive cortical and medullary thymocytes. Double-color analysis by fluorescein-activated cell sorting (FACS) divides these reactive cells into the expected subpopulations: double-positive, double-negative, and single-positive (Fig. 3B). Quantitatively, the representation of each population is similar to that found in a normal, age-matched human fetal thymus (Fig. 3A). In this particular experiment, there were actually more cells with the characteristics of phenotypically mature human T cells, expressing either CD4 or CD8 but not both.

The only histologic difference that we have observed in the SCID-hu thymus is revealed by antibodies to MHC class II antigens. Both normal human fetal thymus and SCID-hu thymus show the expected pattern of human class II antigen distribution (Fig. 2D). The SCID-hu thymus, however, also contains murine class II-positive

cells (Fig. 2E), which have a dendritic morphology and are detectable in the medulla and the mid- and deep cortex. The migratory mechanisms required to produce such microchimerism are apparently not promoted reciprocally. Thus, human T cells and dendritic cells can be observed to move from human fetal liver preparations into the human thymic implant of the SCID-hu mouse, as discussed later, but no human cells are detectable in the host (mouse) thymus with antibodies to the human markers CD2, CD3, CD4, CD8, MHC class I, and MHC class II.

Human T cells in the SCID-hu peripheral circulation. SCID-hu mice with human fetal thymus implants were injected with human fetal liver cells, intravenously or intrathymically. The fate of these cells was then monitored by serial analysis of peripheral blood (Fig. 4). On day 0, four SCID-hu mice with thymic implants (from donor A) were injected intravenously with 10^7 human fetal liver cells (from donor D). Group A/D peripheral blood cells were obtained at intervals of 27, 42, 49, and 64 days thereafter, stained with a mixture of murine monoclonal antibodies to human CD2 (MAb OKT11), CD3 (OKT3), CD4 (OKT4), and CD8 (OKT8), and quantified by FACS. No cells with a human phenotype were observed at day 27 (Fig. 4A). Later, however, such cells were present, representing 17 percent (day 42) and 5 percent (day 49) of total mononuclear cells. By day 64, this population was no longer detectable. Peripheral blood cells from unmanipulated SCID mice were not reactive at any time with this set of antibodies to human T cell markers (Fig. 4B).

Several characteristics are associated with the generation of human T cells in the SCID-hu peripheral circulation. (i) The phenomenon is only observed when the mice have received human thymic implants and human fetal liver cells. The latter, given alone (eight separate experiments to date), do not give rise to phenotypically mature human T cells later. (ii) The timing is predictable (Fig. 5). Whether the fetal liver cells are given intravenously or intrathymically, peripheral human cells are not easily detectable for 3 to 4 weeks. Thereafter, as many as 40 percent of total mononuclear cells in the SCID-hu peripheral circulation may be reactive with antibodies to human T cell markers. In most cases, the circulating human T cells are no longer detectable after 10 to 12 weeks. Variations in kinetics and extent of reconstitution are present. We suspect that this may be dictated by factors related to supply (for example, of stem cells or growth factors or both), to distribution (into body compartments different from peripheral blood), or to changes in human T cell longevity, as, for example, a result of rapid destruction by nonlymphoid murine effector cells.

When the phenotypes were examined immunologically for subset distribution, the T cell subsets in the SCID-hu circulation were similar to those in normal human peripheral blood. In this analysis, peripheral blood cells from SCID-hu mice were stained first with fluorescein isothiocyanate (FITC)-conjugated antibody to HLe-1 (the common human leukocyte antigen, CD45), counterstained with either Texas red-conjugated antibody to CD4 or Texas red-conjugated antibody to CD8, and then quantified with a dual laser FACS. The ratio of CD4 to CD8 (calculated as the percentage of CD4⁺, HLe-1⁺ cells divided by the percentage of CD8⁺, HLe-1⁺ cells) was about 3.8 (standard deviation = 2.0, $n = 23$), a value not dissimilar to that found in normal human cord blood (mean = 4.0 to 5.0) (24). No SCID-hu mice have yet been observed to have ratios of CD4 to CD8, as determined above, of less than one.

Staining patterns in the above experiments are concordant with monoclonal antibodies to different epitopes on the same human T cell marker (for example, OKT4 compared to Leu3a), regardless of whether they are used in two-stage assays (as in Fig. 4) or are directly conjugated. The cells did not stain above background with isotype-matched controls. Finally, within the same mouse, the

human T cell wave is both time-dependent and tissue-restricted. For instance, even when human cells are readily detected in the peripheral blood by FACS analysis, few are found in the mouse spleen and none in the mouse lymph node or thymus (not shown); this might be predicted from the known species-specificity of the lymphocyte homing receptor (25).

To confirm further that the SCID-hu peripheral cells were of human origin, we prepared total cellular DNA from peripheral blood cells and hybridized it with a probe for the human Alu sequence, BLUR 8 (26). Human DNA elicits a strong signal; SCID DNA does not (Fig. 6A). Mice given only human fetal liver cells on day 0 (-/E) show background hybridization by day 35. Littermates given human fetal thymus and human fetal liver cells (A/D) show a strong signal of Alu-hybridizing DNA by that time. The semiquantitative titration suggests that the amount of human DNA in the SCID-hu (A/D) population is about 1 percent of that found in human peripheral blood. Since the number of total mononuclear cells in the SCID peripheral blood is lower than normal, this figure is concordant with the FACS-generated statistic that 10 percent of the mononuclear cells in the A/D group are human. When SCID-hu peripheral blood cells are directly visualized by *in situ* hybridization with an RNA probe to BLUR 8, cells of human origin are found in the midst of unreactive (murine) cells (Fig. 6B). A further correlation with the immunofluorescence data is noted when DNA is analyzed from the peripheral blood of the group F/H/I. This group of mice had received a fetal thymus implant 38 days earlier, fetal liver cells 18 days earlier, and fetal lymph node tissue 11 days earlier. By FACS analysis, the human T cell wave had not yet appeared; by BLUR 8 probing, the human DNA signal is at or only slightly above background.

Human hematopoietic precursors move to and through the SCID-hu thymus. Apparently, the intravenous injection of human fetal liver cells permits T cell progenitors therein to home to and differentiate through the engrafted human fetal thymus. To test this inference directly, fetal liver cells were injected intravenously into a SCID-hu mouse with an MHC-disparate fetal thymus implant (Fig. 7). The fetal liver cells were HLA-A2⁺, HLA-B40⁻; in contrast, the thymic implant was HLA-A2⁻, HLA-B40⁺. Ten weeks after the injection of fetal liver cells, thymic sections were stained with MB40.2, an antibody to HLA-B40, or with MA2.1, an antibody to HLA-A2. As expected (27), MB40.2 was reactive with epithelial cells in the cortex and scattered medullary dendritic cells. No thymocytes were reactive; instead, the densely packed thymocytes in the medulla were HLA-A2⁺. Since cortical thymocytes normally express low levels of class I antigens, definition of their origin by this assay is not possible. However, HLA-A2⁺ dendritic cells are visible in the thymic cortex (Fig. 7B). Thus, progenitor cells to this population must also be capable of movement into the fetal thymus implant.

The subsequent movement of these cells into the peripheral circulation was shown by FACS analysis. Ten weeks after the injection of HLA-A2⁺, HLA-B40⁻ fetal liver cells into a SCID-hu mouse with an HLA-A2⁻, HLA-B40⁺ fetal thymus, cells in the peripheral circulation were double-stained with antibodies to HLe-1 and various class I determinants. No cells in the peripheral circulation of a SCID mouse were reactive with antibodies to HLe-1 or with W6/32, a monoclonal antibody to a monomorphic human class I determinant (Fig. 8A). In the SCID-hu peripheral blood, cells positive for each are detectable (Fig. 8B). Of these, none stain with MB40.2, an antibody to HLA-B40 (Fig. 8C); all stain with MA2.1, a monoclonal antibody to the fetal liver cell type, HLA-A2 (Fig. 8D).

Experiments of a similar nature have been carried out in four groups of SCID-hu mice. In two of these groups, homing of cells to

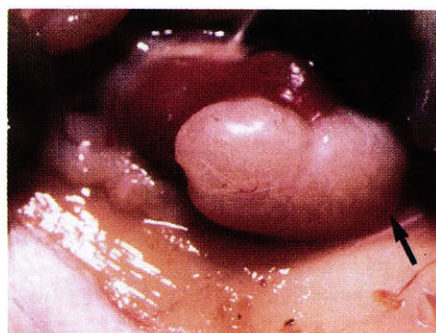
and through the thymus can be demonstrated; homing in the other two groups was not detectable. The apparent complexity of this biology may be related to the relative developmental stages of the thymus or fetal liver cell implants, or to some restriction on the survival of the transplanted cells. It is clear, for instance, that the avian and murine thymic rudiments pass through discrete stages of receptivity for T cell progenitors (3, 28, 29). Whether the human thymus may pass through similar phases during ontogeny remains to be determined.

The problem of T cell function and the presence of human plasma cells. The foregoing studies demonstrate that providing normal human fetal thymus and fetal liver cells to a SCID mouse results in the generation of phenotypically mature human T cells in the peripheral circulation. Dendritic cells of both murine and human origin are also observed to move into the engrafted human fetal thymus. The functional status of these cells is unknown. Put briefly, we observe the following. First, SCID-hu mice do not manifest signs of graft-versus-host disease. Second, they appear to be protected against opportunistic infection. We infer that the human immune system, if functional, has adapted to interact with (or be tolerant of)

murine elements. Possibly, recognition of both murine and human MHC antigens in the thymus has promoted tolerance and restriction to each. This inference requires direct validation.

If human T cells differentiate from human fetal liver cells within the SCID-hu mouse, human B cell progenitors may follow suit. If so, and if the T and B cells were functionally competent, immunoglobulin (Ig) class-switched human plasma cells might be present. The selective binding of protein A to murine and human IgG was used to purify reactive proteins from the plasma of various groups of SCID-hu mice. These proteins were analyzed by SDS-PAGE (Fig. 9A). Human plasma (Hu) contains significant amounts of IgG heavy and light chains; SCID plasma, on the other hand, does not.

Fig. 1. Gross anatomy of the SCID-hu fetal thymus implant.



An autopsy was performed on a SCID-hu mouse, 13 weeks after an implantation of a human fetal thymus under the left kidney capsule; simultaneously, the mouse had received 10^7 fetal liver cells intravenously. Exposed for visualization (cephalad direction to the right) in the left upper quadrant of the SCID-hu abdomen is the SCID left kidney and, growing on it, a human fetal thymus (marked with an arrow). At the time of implantation, the latter represented one lobe of a 9-gestational-week thymus and was less than 0.5 by 0.5 by 2 mm in size. At autopsy, it measured approximately 10 by 5 by 5 mm. The SCID mice were maintained in bonneted isolator cages. They received TMS in suspension through the drinking water for 3 days of each week (40 mg of trimethoprim and 200 mg of sulfamethoxazole per 5 ml of suspension; 0.125 ml of suspension for every 4 ml of drinking water per mouse per day). The drinking water bottle was turned daily while the drug was being administered; standard drinking water was substituted for the remaining 4 days of each week. The gestational age of human fetal tissue was determined (38). Those organs used in the construction of SCID-hu mice were placed in RPMI 1640 medium with 10 percent fetal calf serum (FCS); the remaining tissue was fixed in Formalin. The fetal organs were cut into pieces (2 by 2 by 4 mm). For single cell suspensions, the thymus was pressed between the frosted ends of two glass slides, thereby releasing a suspension of thymocytes. The fetal liver was minced, and cells that remained suspended in the medium were separated into a mononuclear fraction by Ficoll-Hypaque centrifugation; the interface cells were then washed three times in RPMI 1640 with 10 percent FCS and resuspended at a concentration of 10^8 cells per milliliter for intravenous administration. For future use, organ fragments or dissociated cells were frozen in 10 percent dimethyl sulfoxide and 40 percent FCS. For histotyping, thymocytes were incubated first with a panel of monoclonal antibodies to human class I antigens (27), including MA2.1 (IgG₁:A2, B17), PA2.1 (IgG₁:A2, A28), BB7.1 (IgG₁:B7, Bw42), MB40.2 (IgG₁:B7, B40), GAP A3 (IgG_{2a}:A3), MEI (IgG₁:B7, B27, Bw22), and W6/32 (IgG_{2a}:monomorphic), and then with FITC-conjugated goat antiserum to IgG or IgM. The tissues were given a letter code and catalogued with the derived HLA type, gestational age of the fetus, and the presence or absence of fetal anomalies (where known). For implantation of tissues, mice were first anesthetized with halothane. A 1-cm flank incision was made to expose the right or left kidney. The same technique was used to introduce fragments of tissue subcutaneously and into the SCID spleen. Sutures were placed to approximate successive peritoneal and fascial layers, and metal clips were secured over the wound to ensure healing. Suspended fetal liver cells were injected intravenously by a retro-orbital approach, with a 30-gauge needle. [Photograph by F. A. Dusel III]

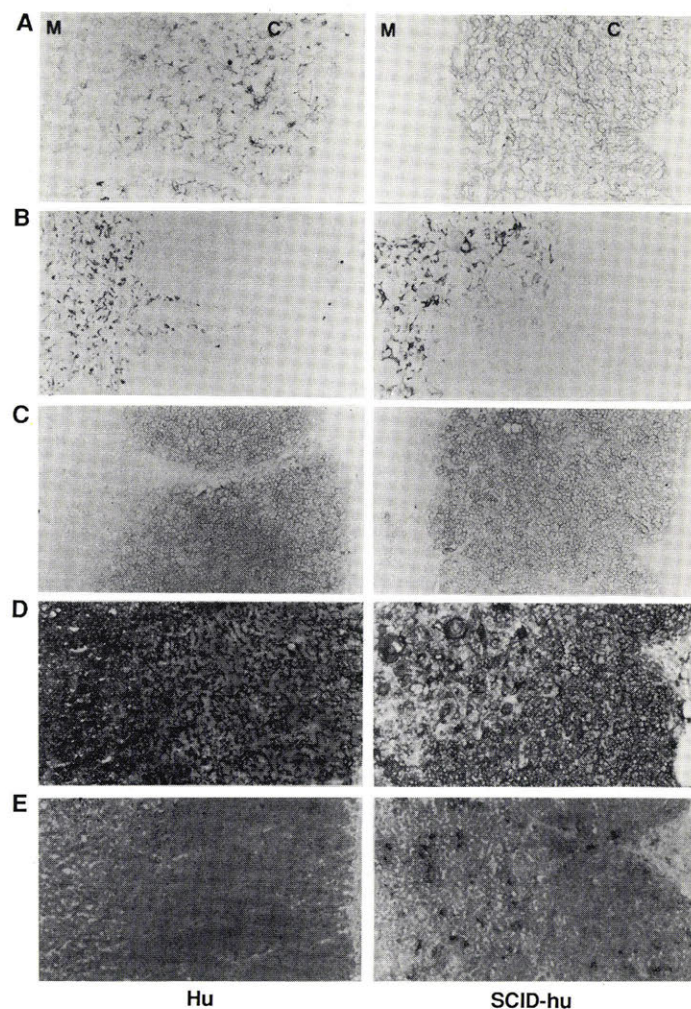


Fig. 2. Microscopic anatomy of the SCID-hu fetal thymus implant. Four weeks after implantation of a 20-gestational week human fetal thymus into SCID-hu group F/H/I (right) (Fig. 9), serial thin sections were prepared. By comparison, sections were also obtained from a normal, age-matched fetal thymus (left). All sections were aligned with the medulla (M) on the left and the cortex (C) on the right. The antibodies used for staining were (A) CDR2, an antibody to human cortical epithelial cells; (B) MD1, an antibody to epithelial cells in the human medulla, in the subcapsular region, and in Hassall's corpuscles; (C) OKT6 (CD1), against cortical human thymocytes; (D) L243, an antibody to a monomorphic determinant on human class II antigens; (E) M5/114.15.2, an antibody to a monomorphic determinant on murine class II antigens. Fresh tissue was dissected into pieces, 3 by 3 by 3 mm, embedded in OCT (Ames, Elkhart, Indiana), quickly frozen in liquid nitrogen, and stored at -70°C . Frozen sections ($6\text{ }\mu\text{m}$) were fixed with acetone for 1 minute at room temperature and then stained by the avidin-biotin-peroxidase complex (ABC) method with the ABC kit (Vector, Burlingame, California) for mouse monoclonal antibodies. For MD1 and M5/114.15.2 (rat monoclonal antibodies), peroxidase-labeled rabbit antibody to rat Ig (Dako, Santa Barbara, California) was used as the secondary antibody. Peroxidase activity was developed by 3,3'-diaminobenzidine.

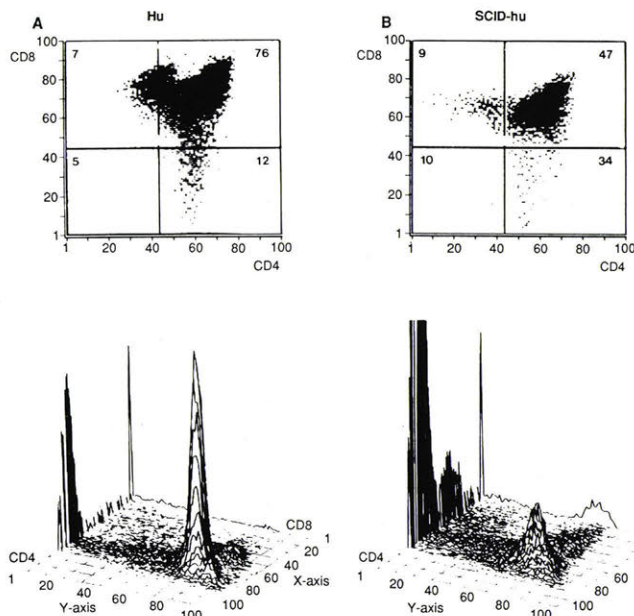
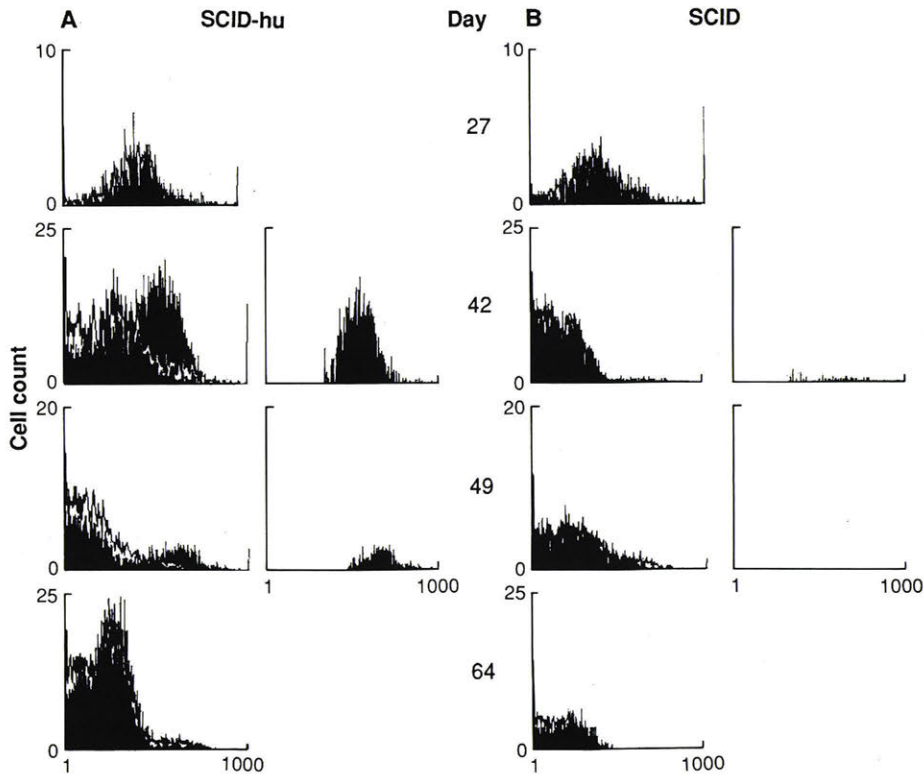


Fig. 3. FACS analysis of SCID-hu thymocytes. The thymic specimens shown in Fig. 2 were prepared as a single-cell suspension, stained with FITC-Leu 3a (CD4) and phycoerythrin (PE)-Leu 2a (CD8), and analyzed by FACS. Two-color profiles of age-matched human thymus are shown on the left (**A**); those of the SCID-hu thymus are on the right (**B**). Subpopulations that are double-negative, single-positive, and double-positive for CD4 and CD8 (and their respective percentage of total thymocytes) are shown in the corresponding quadrants of the top graphs. FITC-Leu 3a and PE-Leu 2a were used as described by the supplier (Becton Dickinson, Mountain View, California).

Fig. 4. Detection of human T cells in the peripheral circulation of SCID-hu mice. SCID-hu mice (group A/D) with a fetal human thymic implant (**A**) were injected intravenously with 10^7 human fetal liver cells (**D**) on day 0. At intervals thereafter, peripheral blood cells were stained with a mixture of monoclonal antibodies to human T cell markers (CD3, CD4, CD8, CD2) and analyzed by FACS (**A**, left panels). For comparison, peripheral blood from an unmanipulated SCID mouse was analyzed in parallel (**B**, right panels). Background staining was assessed with a monoclonal control antibody and is represented by the light curve. Specific staining is shown in the darkened curve. On those days when specific staining was apparent above background in the SCID-hu mice (days 42 and 49), channel per channel subtraction of the two curves was performed to generate the additional plot. After subtraction, 17 percent of the cells stained specifically in the SCID-hu on day 42; 5 percent stained specifically on day 49. Approximately $100 \mu\text{l}$ of whole blood was obtained by tail vein incision and mixed with equal volumes of phosphate-buffered saline (PBS) containing EDTA at 3 mg/ml and of PBS containing 2 percent dextran T-500. Under these conditions, most erythrocytes sediment after 30 minutes at 37°C . Nucleated peripheral blood cells were removed, washed once in RPMI 1640 medium with 10 percent FCS, and then incubated either with a mixture of monoclonal antibodies (OKT3:CD3, OKT4:CD4, OKT8:CD8, OKT11:CD2) or with X63 (an IgG monoclonal antibody to actin). The antibodies to human T cell were used at dilutions previously shown to be optimal for staining; X63 was used at an equivalent protein concentration (1 to 5 $\mu\text{g/ml}$). After 30 to 60 minutes at room temperature, the samples were resuspended, underlayered with undiluted newborn calf serum, centrifuged, and then stained with FITC-antibody to mouse IgG or M (Tago, Inc. Burlingame, California) for 30 to 60 minutes at room temperature. After a



No IgG was seen in the case of the SCID-hu group $-/\text{E}$, which had received human fetal liver cells but not human fetal thymus. However, all other groups of SCID-hu mice (most notably, K/K and F/H/I) were found to make IgG.

The origin of the plasma immunoglobulins was determined by Ouchterlony analysis (Fig. 9B). In conjunction with the data in Fig. 9A, two points are raised:

1) Many of the SCID-hu mice (for instance, K/K, well No. 8) were making readily detectable levels of mouse IgG. SCID mice (unmanipulated littermates) showed low levels of serum IgM, detectable by Ouchterlony and by silver-stained SDS-PAGE; they did not demonstrate detectable levels of IgG. Thus, the provision of human tissues to SCID mice exposes the "leakiness" of the SCID strain (9, 30) and might induce the differentiation of B cells to plasma cells or promote their survival. The progenitors of some of these plasma cells may in turn class switch to IgG.

2) One group of SCID-hu mice, F/H/I, produced human IgG. Using an enzyme-linked immunoabsorbent assay, the amount of IgG in this set of mice was 1 mg/ml, about 10 percent of that found in normal human serum. This set of mice was distinctive in another respect: it was the only group that had been engrafted with human fetal liver cells, human fetal thymus, and also human fetal lymph node. Biopsy specimens derived from that lymph node were prepared for immunohistochemical staining. Its morphology was similar to that of a normal adult lymph node, but cortical and medullary areas were not as well defined (Fig. 10A). The central area of the node was found to contain numerous lymphocytes which bear the CD4 or CD8 markers. By Giemsa stain, plasma cells were seen in the periphery of the node (Fig. 10B). When stained with antibodies to human IgM (Fig. 10C) or human IgG (Fig. 10D), these cells were

final washing, the cells were fixed in 1 percent Formalin and then incubated with propidium iodide (PI) (1 $\mu\text{g/ml}$). This step permitted FACS analysis to proceed quantitatively in the presence of variable numbers of contaminating erythrocytes. Thus, all (Formalin-fixed) nucleated cells were first located by PI staining; mononuclear cells within this population were then analyzed for FITC staining.

Fig. 5. Timing and reproducibility of the human T cell wave in the SCID-hu peripheral circulation. The log percentage of human T cells among total mononuclear cells in the peripheral circulation is shown as a function of time after the intravenous or intrathymic administration of 10^7 human fetal liver cells. The number of mice analyzed at each point is indicated in parentheses. The curve is drawn through the mean of experimental points. These data represent our cumulative experience after 10 months of experimentation. Some SCID-hu mice with human T cells in the periphery for periods as long as 20 weeks have since been noted; the exact conditions which permit such long-term reconstitution are unknown and under evaluation.

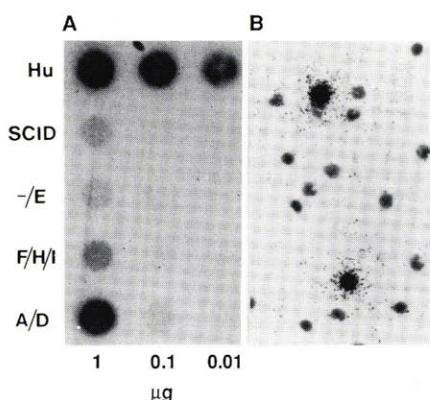
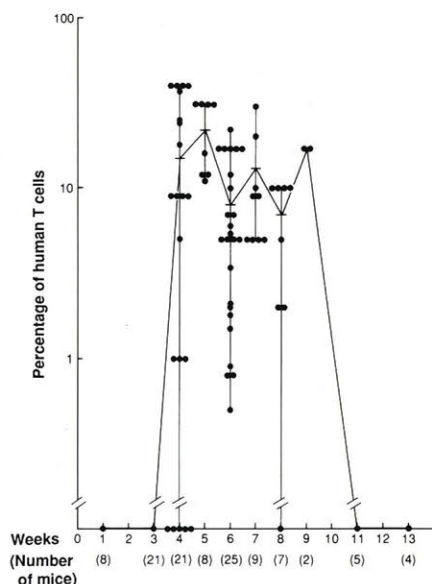


Fig. 6. Hybridization of SCID-hu peripheral blood cells with the human-specific Alu probe, BLUR 8. (A) Total cellular DNA was prepared from the peripheral blood of human (Hu), SCID, and three separate groups of SCID-hu mice: -/E, received fetal liver cells 35 days earlier (day -35), but no fetal thymus; F/H/I, received fetal thymus (day -38), fetal liver cells (day -18), and fetal lymph node (day -11); and

A/D, received fetal thymus (day -61) and fetal liver cells (day -35). (The first two groups of SCID-hu mice showed no staining above background when analyzed on FACS with monoclonal antibodies OKT3, OKT4, OKT8, OKT11; in group A/D, 10 percent of cells were positive.) The DNA was diluted (log-orders), applied to nitrocellulose, and probed with the 32 P-labeled Alu probe, BLUR 8. Nucleated cells were prepared from whole blood (as described in Fig. 4) and lysed in a buffer containing 0.5 percent SDS, 10 mM EDTA, 10 mM tris-HCl (pH 7.4), 10 mM NaCl, and proteinase K at 100 μ g/ml. After 12 hours at 37°C, the lysate was extracted three times with a mixture of phenol, chloroform, and isoamyl alcohol (24:24:1). Total cellular DNA was precipitated from the aqueous phase and diluted in log order (starting at 1 μ g/50 μ l). To each sample was added 30 μ l 2M NaOH and 100 μ l 20 $\times$ SSC (1 $\times$ SSC = 0.15M NaCl, 0.015M sodium citrate); after 10 minutes at 80°C, the sample was neutralized with 320 μ l 1M tris-HCl, pH 7.4, and applied to a nitrocellulose filter in a dot-blotting manifold (Schleicher & Schuell). The filter was baked at reduced pressure for 2 hours, hybridized with the 32 P-labeled Alu probe BLUR 8 under conditions as described (39), and then processed for autoradiography (40). (B) Peripheral blood cells from A/D were prepared for in situ hybridization with a 35 S-labeled RNA probe to BLUR 8. Cells with grains in the autoradiograph are presumably of human origin. Peripheral blood cells were subjected to in situ hybridization as described (41). The RNA probe for BLUR 8 was prepared by subcloning the 0.3-kb Bam HI insert of (plasmid) BLUR 8 into the Bam HI site of pGEM2, to create pGB-8; transcription then proceeded via the T7 promoter in the presence of 35 S-labeled UTP. Autoradiography proceeded for 7 days. Under the conditions used, the probe shows no hybridization to murine spleen cells.

shown to be of human origin. No plasma cells in this lymph node were found to stain with reagents specific for mouse IgG or IgM.

These data indicate that human B cells can differentiate to IgG-secreting plasma cells in the context of a SCID-hu mouse given human fetal thymus, fetal liver cells, and fetal lymph node. Such a differentiative process is normally one in which functional T cells, functional B cells, and functional antigen-presenting cells must interact within a peripheral lymphoid organ; we infer that functional networks are operative, possibly in the lymph node. It is not clear whether T cells derived from the human fetal liver (H) are driving B cell differentiation to plasma cells in the fetal lymph node. (I). Certainly, resident T and B cells in the lymph node at the time of implantation might do the same.

Human hematopoietic differentiation in the SCID-hu mouse.

The SCID-hu mouse represents a murine model system in which human hematopoietic cells of the T and B cell lineages can be observed to differentiate. Differentiation of T cells requires a hematopoietic stem cell source (human fetal liver) and a human fetal thymus. The process appears to require active species-specific homing of intravenously administered cells through the thymus. In a time-dependent and time-limited fashion, phenotypically mature human T cells are then found in the peripheral circulation of the mouse. The rules for human B cell and plasma cell differentiation in the SCID-hu are less certain; the engraftment of intact human lymphoid organs (such as lymph node) appears to be necessary, and perhaps sufficient.

Xenogeneic bone marrow transplantation has been attempted in the past, most notably in the setting of "radiation chimeras" (31-34). Usually, the immune system of the host is quantitatively suppressed by lethal irradiation and allo- or xenoreactive cells in the donor bone marrow are depleted with antibodies to T cells and complement. The results have been variable and, in general, less than encouraging.

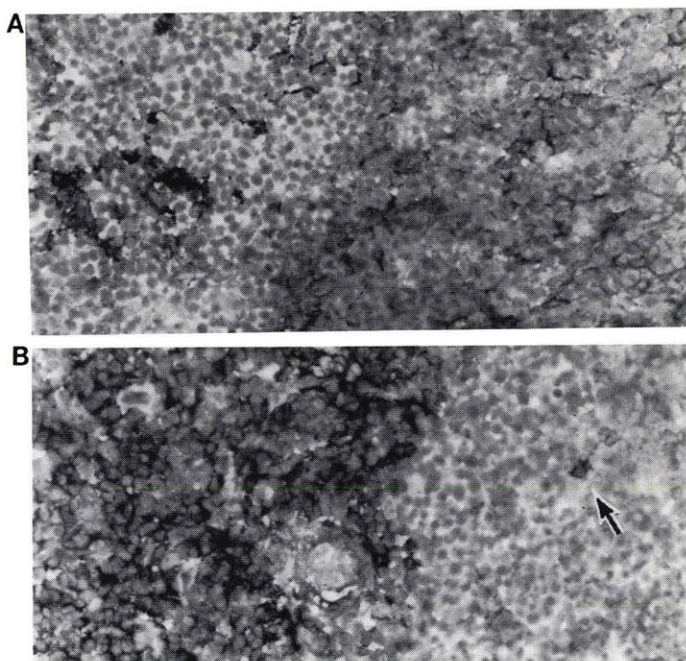


Fig. 7. Movement of human hematopoietic cells from fetal liver preparations to the SCID-hu thymus. A SCID-hu mouse was engrafted with a human fetal thymus (L: HLA-A2⁻, HLA-B40⁺) and injected intravenously with 10^7 fetal liver cells (N: HLA-A2⁺, HLA-B40⁻). Ten weeks later, a biopsy of the thymic implant was sectioned and stained with (A) MB40.2, antibody to HLA-B40, or (B) MA2.1, antibody to HLA-A2. The sections are aligned with the medulla on the left and the cortex on the right. The arrow (in B) points to an HLA-A2⁺ dendritic cell in the host (HLA-A2⁻) thymic cortex. Methods were as described in Fig. 2.

A wasting syndrome (secondary disease) often results and is characterized by varying degrees of immunoincompetence (35, 36). Better results have been obtained with the technique of "mixed chimerism," in which lethally irradiated mice are reconstituted with a mixture of syngeneic and allo- or xenogeneic bone marrow cells depleted of mature T cells (33, 37). This approach has been used recently with success in human bone marrow transplantation. To our knowledge, no reports have appeared in which human → mouse radiation chimeras have been successfully prepared, by any technique.

Our system offers several advantages over the radiation chimera. First, the SCID mouse stock has a selective and total defect in lymphoid cells and does not require preparative irradiation. Breeding colonies are easily maintained with antibiotic (TMS) prophylaxis. Xenografts are implanted without rejection. Second, the use of human fetal tissue obviates the need for T cell depletion. The human T cell progenitor may differentiate to recognize both mouse and human MHC as self. Finally, after engraftment of human tissues, it appears that the SCID-hu mouse is more immunocompetent than its SCID counterpart, not less.

The limitations of the model are obvious. There is variability in the extent and duration of peripheral human T cell reconstitution; at best, it is partial and transient. Further, it is not now clear that the human lymphoid cells are physiologically functional.

Even so, as it stands, the SCID-hu mouse presents new opportunities for the direct analysis of human physiology and pathophysiology. It is reasonable to expect, for example, that the circulating human T cells will present ready targets for infection by human T

lymphotropic viruses. The same is likely to be true of CD4⁺ T cells and myelomonocytic cells in the human lymph node and thymus implants. Possibly, the SCID-hu mouse can serve as a small animal model system for the analysis of diseases caused by human retroviruses. This will most certainly be true for the acute phases of infection, about which we now know the least. Therapeutic agents that might prevent new infection or suppress ongoing infection might likewise be evaluated *in vivo*.

The mature human T cells in the peripheral circulation of the SCID-hu mouse were derived from human fetal liver stem cells. Our evidence suggests that fetal thymus is a necessary intermediate organ. As such, it represents a readout assay for human hematopoi-

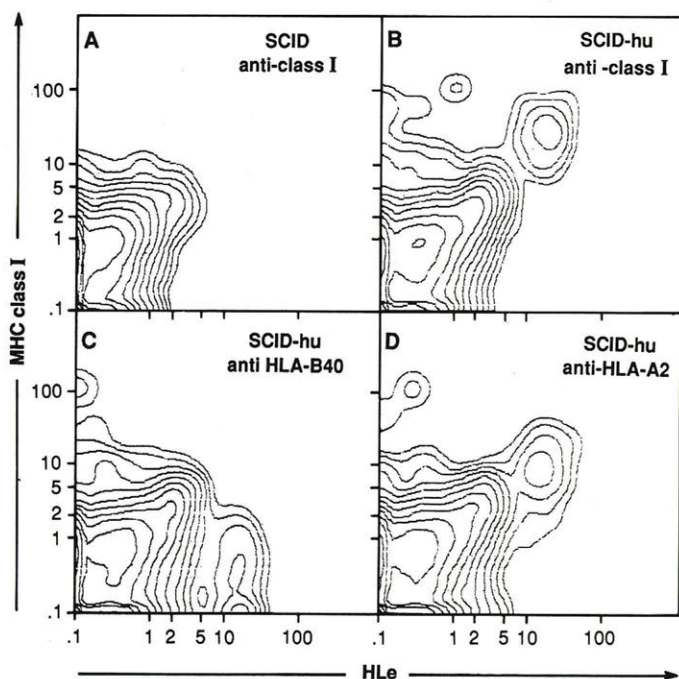


Fig. 8. Movement of human hematopoietic cells from fetal liver cell preparations to the SCID-hu peripheral circulation. Ten weeks after the injection of HLA-A2⁺ fetal liver cells into a SCID-hu mouse with an HLA-B40⁺ thymus (group L/N, see Fig. 7), peripheral blood cells were stained with FITC-labeled antibody to HLe-1 (CD45), a common marker on all human leukocytes. They were counterstained with (B) W6/32, which recognizes a monomorphic determinant on human class I antigens; (C) MB40.2 antibody to HLA-B40; (D) MA2.1, antibody to HLA-A2. These secondary reagents were visualized with the fluorochrome Texas red. As a control, peripheral blood cells from a SCID mouse were reacted with FITC-conjugated antibody to HLe-1 and Texas red W6/32 in (A). Staining procedures were carried out as described (42) with subsequent analysis on a highly modified dual-laser FACS (43).

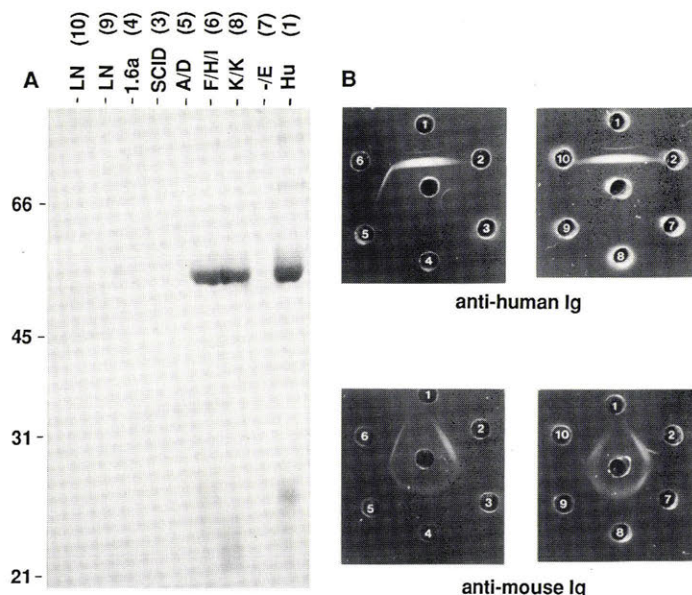
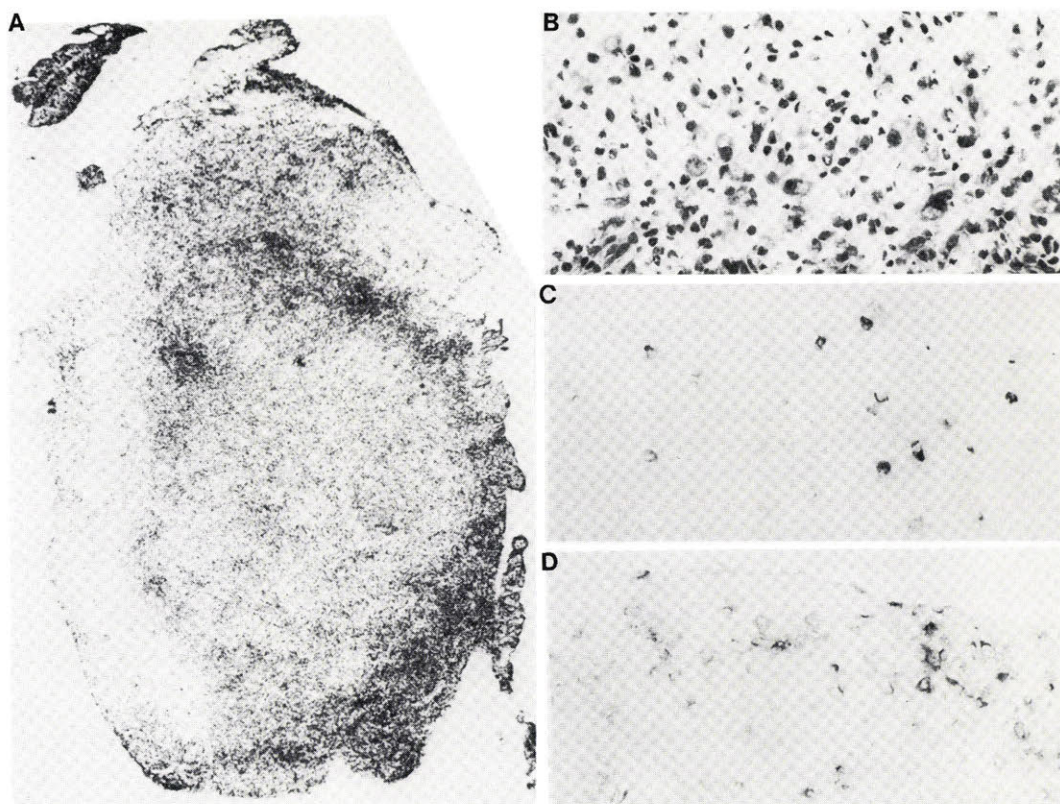


Fig. 9. Protein A-binding proteins in the plasma of SCID-hu mice. (A) Plasma specimens derived from human (Hu), SCID, and seven different groups of SCID-hu mice were incubated with protein A-Sepharose beads. Bound proteins were reduced and alkylated in SDS and resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The gel was stained with Coomassie brilliant blue. The relative mobility of marker proteins is shown on the left. The numbers above each lane correspond to the Ouchterlony wells of (B). Whole blood was collected into an equal volume of PBS containing EDTA (3 mg/ml) and centrifuged at 2000 rpm for 20 minutes to yield plasma. Portions (30 μ l in the case of human, 300 μ l in the case of all other specimens) were incubated with 30 μ l of 50 percent protein A-Sepharose (Pharmacia) for 2 hours at room temperature. The beads were washed, resuspended in an SDS-PAGE loading buffer containing 50 mM dithiothreitol, and prepared for SDS-PAGE (on a 10 percent Laemmli-type gel) (44), as described (40). The gel was subsequently stained in 0.125 percent Coomassie brilliant blue. (B) The same plasma specimens were placed on equal portions around central wells of Ouchterlony plates containing antibody to human Ig (top) or antibody to mouse Ig (bottom). The description of each SCID-hu (except 1.6a) is given as follows: X/Y/Z (X: gestational age of human fetal thymus at time of implantation; number of days since implantation. Y: gestational age of whole human fetal liver or fetal liver cell suspension; number of days since implantation. Z: gestational age of human fetal lymph node at time of implantation; number of days since implantation). (1) Human plasma; (2) BALB/c plasma; (3) SCID plasma; (4) SCID-hu plasma, 1.6a (received a serial transplant of fetal thymus from the thymus shown in Fig. 2, 170 days before assay; and fetal liver cells intravenously 140 days prior to assay); (5) SCID-hu plasma, A/D (A: 22 weeks, 96 days since implant/D: 22 weeks; 90 days since implant); (6) SCID-hu plasma, F/H/I (F: 20 weeks; 68 days since implant/H: 22 weeks; 48 days since implant/I: 15 weeks; 41 days since implant); (7) SCID-hu plasma, -/E (-: no fetal thymus/E: 19 weeks; 90 days since implant); (8) SCID-hu plasma, K/K (K: 20 weeks; 30 days since implant/K: 20 weeks; 30 days since implant); (9) SCID-hu plasma, L/N (L: 19 weeks; 20 days since implant/N: liver, 19 weeks; 20 days since implant); (10) SCID-hu plasma, L/N (L: 19 weeks; 20 days since implant/N: bone marrow, 19 weeks; 20 days since implant).

Fig. 10. Human plasma cells in the fetal lymph node of a SCID-hu mouse. Serial thin sections were prepared from a SCID-hu fetal lymph node 7 weeks after implantation of a 15-gestational-week human fetal lymph node into group F/H/I. The gross morphology is shown by Giemsa stain in frame (A); under higher magnification, cells with the morphology of plasma cells are seen (B). Lymph node cells are found to be reactive with antibodies against human IgM (C) and human IgG (D). No reactive cells are seen with antibodies to murine immunoglobulins. Peroxidase-conjugated primary antibodies were used in this experiment, as described in Fig. 2. The samples in (A) and (B) were Formalin-fixed; the frozen sections in (C) and (D) were acetone-fixed.



etic stem cells. A number of markers may be used to trace the movement of certain human cells into and through the thymus, for example, MHC types (as in these experiments), Y chromosomal markers, and chromosomal abnormalities. These markers, combined with selected monoclonal antibodies and FACS, should prove to make the SCID-hu a useful model for the description and isolation of the human pluripotent hematopoietic stem cell, as well as lineage-restricted progenitors.

When fetal liver cells, fetal thymus, and fetal lymph node are implanted in SCID mice, B cell differentiation to IgG-secreting plasma cells occurs. Much more needs to be learned about this set of events. It is possible that these developing cells might be specifically sensitized in vivo. If so, the SCID-hu mouse may thereby constitute a vehicle for the generation of human monoclonal antibodies.

Finally, the SCID mouse appears to present no barrier to transplantation of tissues from single or multiple human donors. Pathologic human tissues might be transplanted also, and thus be available for the direct study of human physiology and pathology in an experimental animal.

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Infection of the SCID-hu Mouse by HIV-1

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Infection of the SCID-hu Mouse by HIV-1

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J. M. McCUNE*

SCID-hu mice with human fetal thymic or lymph node implants were inoculated with the cloned human immunodeficiency virus-1 isolate, HIV-1_{JR-CSF}. In a time- and dose-dependent fashion, viral replication spread within the human lymphoid organs. Combination immunohistochemistry and *in situ* hybridization revealed only viral RNA transcripts in most infected cells, but some cells had both detectable viral transcripts and viral protein. Infected cells were always more apparent in the medulla than in the cortex of the thymus. These studies demonstrate that an acute infection of human lymphoid organs with HIV-1 can be followed in the SCID-hu mouse.

MULTIPLE LINES OF EVIDENCE suggest that the etiologic agent of the acquired immunodeficiency syndrome (AIDS) is human immunodeficiency virus (HIV) (1). Definitive proof and direct analysis of infection by HIV *in vivo* has been hampered by the lack of a suitable small animal model. Thus, we know little about the events which occur immediately after infection or about the subsequent progression of disease (2). A number of experimental animal models have been advanced. All are approximations with severe limitations: either an animal retrovirus other than HIV is used to infect a host other than man (3), or HIV is used in a context beyond that of its natural tropism (for example, in primates or rabbits) (4). The ideal system would allow the study of the interactions that occur during the natural course of disease, that is, those of HIV with the human lymphoid system. The SCID-hu mouse (5) was constructed precisely for this purpose. We present evidence now that this mouse model supports infection by and replication of HIV-1.

Implantation, growth, and interactions between the human lymphoid organs of the SCID-hu mouse have been described (5). In many respects, the environment of this hematochimeric mouse is similar to that encountered by HIV in man. After parenteral

inoculation or sexual contact, it seems likely that HIV drains to and is sequestered within lymphoid organs such as lymph nodes, Peyer's patches, or spleen; during perinatal infection, the developing thymus may also be a major target organ for infection (6). These lymphoid organs present full complements of interactive cells which are permissive for infection, including CD4⁺ T cells and myelomonocytic cells. The human fetal thymus and lymph node implants which grow in the SCID-hu mouse have microscopic features and cellular compositions that are essentially indistinguishable from those of their normal human counterparts (5). We anticipated that parenteral inoculation of these organs would provide a previously unobtainable view of acute infection with HIV *in vivo*.

A molecular clone of HIV-1 was used in these studies (7). Derived from the cell-free cerebrospinal fluid of a patient with subacute encephalopathy and Kaposi's sarcoma,

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HIV-1_{JR-CSF} was propagated in short-term culture with phytohemagglutinin-stimulated human peripheral blood lymphocytes and then cloned into the bacteriophage vector EMBL4 (8). This isolate is infectious for mitogen-stimulated human T cell blasts and for glial cell explants, but not for primary monocyte-macrophage cultures. It has not been passaged and does not grow in continuous human T or monocyte-macrophage cell lines (H9, Jurkat, MOLT4, HUT-78, U937). Such tropism marks a potentially important difference between it and other isolates which have been cloned and adapted to growth in vitro (for example, LAV, HTLV-III_B, HXB2, and ARV) (9).

SCID-hu mice were infected within the confines of a specially adapted animal bio-safety level (BSL) 3 facility (10). A flank incision exposed the growing human thymus or lymph node implant of anesthetized SCID-hu mice. Graded doses of HIV-1_{JR-CSF} were introduced by direct intrathymic or intranodal inoculation. The mice were maintained in micro-isolator cages, inside a sealed glove box, within the BSL3

facility. At various time points, biopsy specimens of the infected lymphoid organs were obtained, fixed in 4% paraformaldehyde, and assayed for signs of acute infection.

Inoculation of SCID-hu mice with HIV-1_{JR-CSF} generated detectable signs of viral replication. The most direct assay was in situ hybridization of infected tissue sections with the RNA probe pG4 (Fig. 1) (11). This probe hybridizes to the 3' end of the genomic HIV-1 transcript (see legend to Fig. 1) accordingly, all viral RNA transcripts (genomic and subgenomic) are detected without discrimination. Under the conditions of the assay, viral DNA is not detected. Infection of the human thymus implants was time-dependent. At 1 week after intrathymic inoculation of 400 to 4000 infectious units (IU), no cells in the section hybridized with the pG4 probe (Fig. 1A). At 2, 4, and 8 weeks (Fig. 1, B, C, and D, respectively), more cells hybridized (see below, Fig. 3). This suggests that ongoing rounds of infection occurred in vivo. The infected cells were scattered throughout the cortex and medulla of the injected human thymus and

across the injected human lymph node (Fig. 1F). Discrete foci of infection, involving more than one contiguous cell, were only rarely observed (see below, Fig. 2B).

The events described in Fig. 1 are specific for HIV-1 infection. In infected tissue no cells hybridized with pG4 RNA probes in the "sense" orientation (Fig. 1E); no cells in uninfected tissues hybridized to pG4 probes in either orientation. Thymic or lymph node implants inoculated with heat-inactivated virus (80°C, 1 hour) did not hybridize after 4 weeks. The infection was also dose-dependent. Thus, a 10⁻¹ dilution of HIV-1_{JR-CSF} was infectious, but dilutions of 10⁻³ and 10⁻⁵ were not. Using estimates of viral titers determined by in vitro assays, the 10⁻¹ input dose corresponded to 400 to 4000 IU. (12).

We also applied immunohistochemical stains to the tissue sections. The polyclonal antiserum GB, selected for its high-titer reactivity with viral env, gag, and pol determinants, was used. Many cells in the infected thymus were reactive with this antiserum. In all cases examined, the number of reactive cells was fewer than the number reactive by in situ hybridization with the pG4 probe (11). To explore this observation further, thymus tissue sections 2 weeks after HIV-1_{JR-CSF} infection were stained first with immunohistochemical stains and then by in situ hybridization (Fig. 2). In this manner, the presence of viral protein could be detected in cells making viral RNA transcripts; in addition, the cell surface phenotype of infected cells could be determined. In both the medulla and the cortex of the thymus, as well as in the lymph node, cells were found to produce either detectable amounts of viral protein and RNA (arrows, Fig. 2A) or only viral RNA transcripts (all other reactive cells, Fig. 2A). The latter population represented the majority of total infected cells in either compartment (Fig. 3). No cells were observed to produce only viral protein in the absence of detectable viral RNA. The basis for this observation is not clear. An artifact related to the procedure itself appears unlikely: cells with each mode of expression (viral RNA and protein, or viral RNA alone) are randomly dispersed and frequently adjacent (see Fig. 2, A and B). More likely, patterns of transcriptional and translational control after infection may differ in distinct cell types, differentiative stages, or both. Complex patterns of this sort have been documented in vivo after infection with other lentiviruses (for example, visna virus and canine arthritis-encephalitis virus) (13). Their existence is consistent with recent experiments on HIV-1 in vitro (14). The regulatory mechanisms by which they occur may now be explored with HIV-1 in

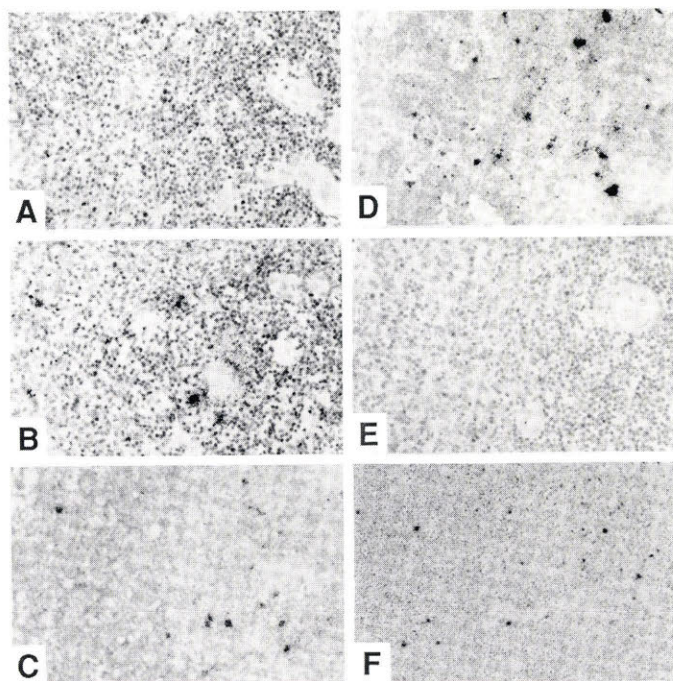


Fig. 1. Infection of the SCID-hu fetal thymus and lymph node implants with HIV-1. Eight weeks after implantation of human fetal thymus (A to E) or lymph node (F) into SCID mice, 400 to 4000 IU of HIV-1_{JR-CSF} (in 20 μ l) were introduced by direct intrathymic or intranodal injection. At intervals of 1 week (A), 2 weeks (B, E, and F), 4 weeks (C), or 8 weeks (D) thereafter, the implants were harvested, fixed in 4% paraformaldehyde in phosphate-buffered saline at 4°C for 4 to 8 hours, washed with phosphate-buffered saline for 24 hours, and then frozen in O.C.T. embedding compound (Miles Laboratories, Inc.). Six-micrometer sections were subjected to in situ hybridization (15) with a ³⁵S-labeled antisense (A to D and F) or sense (E) RNA probe to the 3' end of HIV-1, pG4. This probe was prepared by subcloning the 1.1-kb Bam HI-Hind III fragment of HXB2 [nucleotides 8054 to 9199, according to (16)] into the multicloning site of pGEM1. Transcription was from the SP6 promoter (anti-sense, A to D and F) or T7 promoter (sense, E) in the presence of ³⁵S-labeled UTP. Under the conditions used, neither probe hybridized to uninfected mouse or human cells, or to HIV-1 DNA; therefore, cells with grains in the autoradiograph are presumed to be infected with transcriptionally active HIV-1. All micrographs of thymus (A to E) are aligned such that the medulla is on the right and the cortex on the left.

Fig. 2. Simultaneous visualization of HIV-1 viral protein and RNA within infected SCID-hu thymus. A SCID-hu mouse was infected by intrathymic injection of HIV-1_{JR-CSF} (400 to 4000 IU). Individual 6- μ m thymic sections were analyzed 2 weeks later by successive applications of immunohistochemical stains and in situ hybridization. They were first immunostained with the human polyclonal antiserum GB (1:300 dilution) and then with peroxidase-labeled goat antibody to human immunoglobulin G (1:100) (CALTAG Laboratories). The peroxidase activity was developed with 3-amino-9-ethylcarbazole. Second-stage antibody alone was unreactive with infected thymus. In uninfected thymus, no reaction products were seen using this combination of antibodies. The immunostained tissue sections were then fixed again with 4% paraformaldehyde (20 min, 20°C), and subjected to in situ hybridization with the antisense pG4 RNA probe (as described in Fig. 1). Representative areas are shown of thymus medulla (A) and cortex (C). Cells reactive with both antibody and RNA probes are marked by arrows in (A). Medullary cells of the appearance shown in (B) were occasionally observed; at all times, they showed evidence of both viral protein and RNA.

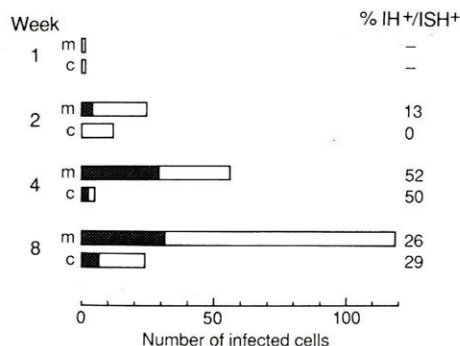
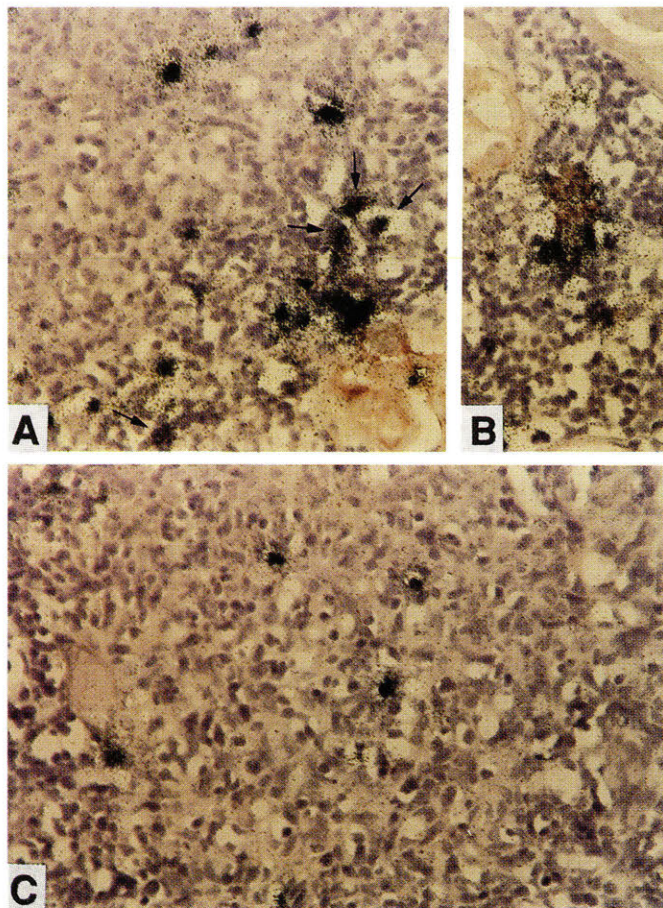


Fig. 3. Quantitation of HIV-1 infection of the SCID-hu thymic cortex and medulla. The SCID-hu thymic sections of Fig. 1 were subjected to immunohistochemistry and in situ hybridization in parallel to detect HIV-1 viral protein and RNA transcripts. At each time point, two observers counted the number of cells in five to ten high-power (400 $\times$) fields that stained with the antibody GB or with the RNA probe pG4. The bar graphs represent the mean number of infected cells in either the thymus medulla (m) or cortex (c) that were detected by in situ hybridization. The mean number of cells that stained with antibodies to HIV-1 is shown as a darkened area of each plot. To the right, the number of cells immunohistochemically stained (IH) is expressed as a percentage of the number of cells positive by in situ hybridization (ISH): %IH+/ISH+.

vivo, within the SCID-hu mouse.

More cells were found to be infected in the medulla (Fig. 2, A and B) than in the cortex (Fig. 2C), even though there was an abundant representation of CD4⁺ thymocytes in each compartment (5). A similar situation was found at weeks 4 and 8 after infection, throughout multiple planes of observation (Fig. 3). Thus, within any given section at any given time, 70 to 90% of the infected cells were medullary in location. Multinucleated giant cells, a common feature of HIV infection in vitro, were occasionally seen (Fig. 2B).

In summary, human thymus and lymph node transplanted into SCID mice support the in vivo initiation and spread of infection by HIV. The SCID-hu mouse thus appears to provide a fertile environment for further investigations into the course of infection by HIV in vivo. To the extent that one may observe the onset of infection within complex lymphoid organ systems following parenteral inoculation with HIV, it should be possible to dissect subsequent interactions systematically, after infection, on both a cellular and molecular level. Such analyses,

initiated in vivo within a small animal model with human lymphoid organs, might more closely approximate the course of infection in man and more readily lead to meaningful and useful paradigms.

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11. Similar results to those shown in Figs. 1 and 3 were obtained with another RNA probe, pG3. This probe was constructed in pGEM1 by subcloning the Hind III-Bgl II fragment between nucleotides 5610 and 6622 of HXB2 (16); it should predictably hybridize to viral genomic RNA and to envelope mRNA.
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SYSTEMIX



Company Profile

Utilizing SCID-hu technology for
basic research and product development
related to human disease states

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April 1989

The rational treatment of human illness requires a fundamental understanding of disease processes. Without such understanding, the development of new therapies becomes a hit-or-miss proposition that is costly, time-consuming and often unfruitful. This may seem an obvious precept; but as many clinical researchers will attest, key data is often not obtainable.

In the search for new human therapies, scientists traditionally have had to rely on studies in cultured laboratory cells and animal models. However, information provided by these studies frequently has limited value in answering questions about the progression of illness or a product's therapeutic potential in humans. In many cases, human clinical tests must be conducted before the needed insight has been obtained, and the researchers can only hope to draw conclusions about specific disease processes from test results. Often, critical information remains unknown, hampering the treatment of numerous life-threatening illnesses, including cancer and acquired immune deficiency syndrome (AIDS).

Now, finally, there is a suitable experimental model for studying human diseases: the SCID-hu mouse. As reported in the September 23, 1988 issue of Science, this remarkable new animal model is constructed by the engraftment of human hematopoietic stem cells, thymus and lymph nodes into a mouse that does not have a functioning immune system of its own. The resulting chimeric animal, the SCID-hu mouse, accepts and stably maintains the transplanted tissue in an interactive and apparently functional manner. As such, it may help solve major research questions.

Initially developed by scientists at Stanford University, the SCID-hu mouse has been licensed exclusively to SyStemix, Inc. The company is now in the process of standardizing this revolutionary experimental model and compiling extensive research data.

Through internal studies and collaborative relationships with research organizations throughout the world, SyStemix intends to utilize the SCID-hu mouse as a research tool. The ultimate goals are as follows:

- To understand the pathology of human diseases.
- To evaluate products under development.
- To create new products to overcome serious illnesses.
- To increase knowledge about the human immune system.

SyStemix is a California-based biomedical company founded in mid-1988 by four scientists interested in moving important basic research forward into therapeutic applications. The company's founders include:

- J. Mike McCune, M.D., Ph.D., the immunologist who invented the SCID-hu mouse model.
- Irving L. Weissman, M.D., a widely recognized pathologist working on hematopoietic differentiation, viral oncogenesis and cellular immunology.
- David Baltimore, Ph.D., a Nobel laureate specializing in research related to molecular virology and mechanisms of eukaryotic gene activation.
- Leroy E. Hood, M.D., Ph.D., a renowned expert in immunogenetics, immunochemistry and cellular immunology.

Together they have assembled at SyStemix an extraordinary team of M.D. and Ph.D. researchers skilled in cellular immunology, immunochemistry, retrovirology, molecular biology, microbiology, developmental biology, pathology, immunopathology, protein chemistry, complex analytical techniques, and other fields important to the study of disease processes and treatment responses.

Following a carefully-conceived plan for the commercial development of SyStemix, their scientific efforts are focused on three primary objectives.

- In the near term — conducting independent and collaborative research on disease pathology and/or physiology in the SCID-hu mouse. Plans include:
 - Creation of an animal testing facility focused on AIDS and other infectious diseases.
 - Evaluation of new drugs and vaccines to suppress and/or prevent AIDS and other viral infections.
- In the intermediate term — developing specialized SCID-hu models to study a range of human diseases and utilizing these models to develop products for SyStemix and for other organizations.
 - Of particular interest are therapeutic and diagnostic applications to address cancer, infectious diseases, autoimmune diseases and other conditions involving the immune system.
 - More specifically, the company will focus on creating human (as opposed to mouse) monoclonal antibodies for therapeutic use.

- In the long term — independently exploring new opportunities and constructing human products with the aid of SCID-hu mice. For example:
 - Purified stem cells to enhance bone marrow transplantation.
 - Growth factors to proliferate stem cells and serve as new therapies.

The company's aim is to carefully manage its expansion, while simultaneously recognizing the importance of making SCID-hu mice available for productive areas of research as quickly as possible.

SyStemix currently occupies 24,000 square feet of research and administrative space in Palo Alto, California. By year-end, the company expects to have built a prototype infectious disease testing facility. This extensive BL3 containment facility will incorporate unique animal handling and analytical equipment suitable for the safe study of serious infectious pathogens.

From a business perspective, SyStemix has a strong management team, supported by a highly experienced Board of Directors and a very active, well connected Scientific Advisory Board. The company is in a solid financial position, with \$10 million in initial funding provided primarily by E.S. Jacobs & Company and Aetna Life Insurance Company. Additional funding is expected from strategic alliances, as well as from the formation of a consortium of companies that will support creation of the infectious disease testing facility.

The SCID-hu mouse presents an entirely new paradigm for biomedical research. Unlike any other animal model, it was created precisely for the study of the human immune system, its physiology and its pathophysiology. As such, the SCID-hu mouse could revolutionize the testing of potential drugs and vaccines, and could help overcome immune system barriers that currently limit transplantation of many tissues and organs.

SyStemix now has a colony of more than 800 SCID-hu mice. These experimental animals are engrafted with human tissues that are responsible for development of the complete human immune system. They are the result of complex proprietary techniques allowing human tissue of interest to be segregated, transplanted and explored within the physiologic confines of immune deficient mice called SCID (for severe combined immune deficiency). Substantiating the effectiveness of this approach, SCID mice normally fall victim to a fatal opportunistic infection, *Pneumocystis carinii* pneumonia; but after transformation into SCID-hu mice, they appear to acquire immune defenses against this disease.

Each mouse receives human fetal liver (containing hematopoietic stem cells), fetal thymus and fetal lymph nodes. These tissues are implanted in such a manner that a functional immune system is created, representing all major classes of lymphocytes, including a full complement of T cell subsets. (See "The SCID-hu Mouse," McCune, et al, Science, vol. 241, pp. 1632-1639, September 23, 1988.) Ultimately, the company expects to identify other human organs — such as skin, kidney, pancreas, reproductive and neuronal tissue — that can also function in this unique animal.

Prior to the development of the SCID-hu model, there was no *in vivo* method for rationally and systematically exploring the human immune system. While animals, including primates, are regularly used in disease studies, the relevance of the test results are often uncertain. In the absence of a representative animal model, human clinical studies have become the only available means of evaluating many therapeutic and prophylactic modalities. However, due to the ethical limits of human experimentation, such studies have provided little insight into disease processes.

In contrast, the SCID-hu mouse model represents a straightforward method for developing relevant data. This unique model is simply constructed,

easily controlled and amenable to manipulations consistent with the humane care of laboratory animals. The problems that may be approached within it involve human diseases and human cells; the research answers are therefore more likely be directly applicable to human beings.

The SCID-hu model can be used today for certain types of studies, and is being standardized for broader applicability. In addition, since human-specific pathogens can be introduced into SCID-hu mice, disease-specific models are under development for illnesses caused by infectious agents such as the human immunodeficiency virus (HIV), which causes AIDS, and the human T cell leukemia virus (HTLV-1), which causes cancer. Additional disease models will be created as conditions warrant.

SyStemix expects that within the coming months SCID-hu mice will be used to address a wide range of efficacy, dosage and toxicology questions related to various products under development. Information provided by the SCID-hu studies can be used to fine-tune development efforts, to guide the design of human clinical studies, and, hopefully, to quicken the entry of important new therapeutics and diagnostics into the marketplace.

Tests indicate that the SCID-hu is a versatile model of the human immune system. Human T cell and B cell lineages have already been specifically isolated in the SCID-hu. Within the conceived limitations of the model, there is every reason to believe that other lineages of human hematopoietic ("blood-forming") stem cells can be equally well isolated for study. The model is also amenable to the isolation, definition and manipulation of relevant stromal cells and growth factors. (Stromal cells normally produce growth factors to drive the regeneration or differentiation of each of the stem cell lineages.) Furthermore, SCID-hu mice can be used as bioassays for the proliferation and differentiation of human stem cells, giving rise to the possibility of creating preparations of purified stem cells for safer, more effective bone marrow transplantation. Additional research indicates that human plasma cells can be specifically sensitized *in vivo* (within the mice), providing a potential vehicle for generating human monoclonal antibodies with therapeutic applications.

The following pages describe the near-term strategies that SyStemix is implementing with regard to the SCID-hu mice.

The emergence of AIDS underscores the need for direct and comprehensive analysis of the human immune system. Diseases associated with human lymphotropic retroviruses, such as HIV, are difficult to study. Much still needs to be learned about the course of HIV infection in man, and the accumulation of knowledge is hampered by the limitations inherent in traditional clinical research approaches. Although similar retroviruses do exist in animals, none are identical to the human virus and none produce comparable animal diseases. The development of effective therapies is therefore a difficult and time-consuming process.

The SCID-hu model could dramatically change this situation. Not only do these mice offer a window to the human immune system; but they also permit infection with HIV. Therefore, in much the same manner as the lymphoid organs in man are likely to be infected by the virus, the engrafted human thymus and lymph nodes are attacked in the SCID-hu mouse. (See "HIV Infection of the SCID-hu Mouse," Namikawa, et al, Science, vol. 242, pp. 1684-1686, December 23, 1988.)

By thus providing a suitable small-animal model of HIV infection, the SCID-hu mice may enable scientists to finally determine exactly where in the body and by what means the AIDS virus causes disease. In addition, for the first time, scientists will be able to evaluate new drugs or vaccines in the setting of an experimentally-controlled HIV infection of human organs. Questions related to route of administration, dose, volume of distribution, metabolism and efficacy may be addressed in such a way as to yield meaningful results.

The SCID-hu model of HIV infection is now being more fully developed and standardized by SyStemix. Moreover, plans have been made to create conditions under which numerous drugs or vaccines can be tested efficiently and effectively against not only HIV but other highly infectious agents as well.

A top priority for SyStemix is therefore the creation of an animal testing facility focused on HIV and other viral infections. The laboratory will utilize the SCID-hu mice, as the first true animal models for AIDS research, and it will operate in collaboration with research organizations worldwide. SyStemix expects to fund the development of this GLP facility through a specially-created consortium of pharmaceutical and biotechnology companies.

The following plan will be implemented by SyStemix for this unique animal testing facility:

- The laboratory will house large numbers of fully standardized SCID-hu mice, along with a computerized body of knowledge on the mice and their responses to HIV infection.
- The services of the laboratory will be available on a collaborative basis to other companies, academic scientists and medical research institutes already engaged in AIDS research and/or product development.
- The SCID-hu will serve as a screening and testing tool, providing data important to the creation of safe, effective drugs and vaccines:
 - To prevent the initiation and/or spread of HIV infection.
 - To suppress and/or eliminate opportunistic infections, AIDS-related cancer (such as Kaposi's sarcoma and leukemia), and other life-threatening conditions.
- In addition to product-oriented experiments, basic discovery research will be conducted:
 - To explore HIV and determine the precise mechanisms by which the retrovirus gains a stronghold in the human body.
 - To investigate the course of AIDS and the progressive destruction of the human immune system.
 - To identify the cell types involved during the course of disease, including those infected by HIV as well as those which react to the infection.

Because of their broad applicability, SCID-hu mice make ideal models for studying the still-unknown ways in which a number of serious diseases progress. In fact, as long as the target organ for a particular disease can be propagated in the SCID-hu mice, the pathophysiology of the disease, as well as the human immune response, can be studied and subjected to experimental manipulation. Moreover, researchers can utilize the SCID-hu mice to help guide product development efforts and direct various choices regarding formulation, dosage levels, the staging of treatment, etc. Such work could, in turn, speed the commercialization of new types of vaccines, neutralizing antibodies and other innovative pharmaceuticals.

SyStemix will form joint ventures, R&D partnerships and other collaborative relationships with biotechnology, pharmaceutical and diagnostic companies, as well as academic and government research institutes. The goal of such relationships will be to study the pathogenesis of specific diseases and the use of existing drugs against these diseases, as well as the safety and efficacy of new drugs in development. Basic research and product development opportunities include:

- Infectious diseases caused by pathogens that cannot be adequately studied in conventional animal models — for example, diseases resulting from infection by HTLV-1, type I and type II herpes viruses, cytomegalovirus, Epstein-Barr virus, human papillomaviruses, and the hepatitis A, B and non-A/non-B viruses.
- The study of major histocompatibility complex (MHC) restriction and tolerance, through the use of MHC-restricted human cytotoxic T cells produced in SCID-hu mice.
- Graft versus host disease and prevention of organ transplant rejection.
- Cancer of the blood and immune system, including leukemia and lymphoma.
- Solid tumors, such as breast cancer, associated with oncogenic proteins that may impact the progression from early stage cancer to fatal disease.
- Autoimmune diseases, such as rheumatoid arthritis, in which the body's immune system has turned against itself.
- *In vivo* effects of therapeutics on the human immune system, including the effects of human cytokines (such as the interleukins and growth factors), as well as more conventional drugs.

It is currently impossible to generate specific primary human immune responses in a predictable manner. As a result, human monoclonal antibodies against many disease-producing agents cannot be easily made. However, scientists now have the opportunity to immunize and stimulate a human IgG response in SCID-hu mice. SyStemix intends to explore this opportunity further, developing and licensing techniques for producing human monoclonal antibodies (as opposed to mouse antibodies) for therapeutic applications.

The company's aim is to optimize conditions in the SCID-hu mouse, whereby specific human IgG responses can be created to any given antigen and immortalized in hybridoma cell lines. This application of SyStemix technology would appear to allow production of human antibodies that have distinctive classes (for instance, IgG and IgA), selectable affinity, and human Ig heavy chain allotypes that are most appropriate for patient care.

The company intends to develop specific human monoclonal antibodies against carefully selected antigens of medical importance. Concurrently, SyStemix will enter into partnerships with other enterprises interested in using the company's technologies to generate their own human monoclonal antibodies against selected antigens.

Among the possibilities are monoclonal antibodies against the following:

- Infectious agents — gram-negative bacteria, pneumococcus, influenza A, cytomegalovirus, Epstein-Barr virus, HIV, HTLV-1, hepatitis A, B and non-A/non-B.
- Blood group antigens — MHC complex allotypes (Class I and Class II), Rh factors.
- Tumor-specific markers — idiotypes, human melanoma antigens, colorectal tumor antigens.
- Mediators of inflammation — TNF, IL-1, interferon gamma, endotoxins.

Until the advent of the SCID-hu mouse, work with human hematopoietic stem cells and their associated growth factors has been limited to *in vitro* culture systems of uncertain relevance and to clinical studies in man. As a result, many critical questions remain unresolved. SyStemix has the methodology to conduct basic research on this important area of the immune system and reveal the answers to many pertinent questions.

Hematopoietic stem cells are the "parent cells" of all the varied cells of the blood and immune system — including erythrocytes or red blood corpuscles, leukocytes or white blood corpuscles, and platelets. Some stem cells (the "pluripotent" cells) give rise to all lineages, while others (the "lineage-restricted" cells) give rise to selected lineages, such as the lymphoid, myelomonocytic, erythroid and megakaryocytic series of blood cells. These cells form in the fetal liver and in both the fetal and adult bone marrow. They then migrate to the thymus gland, the lymph nodes and other organs.

Mouse bone marrow hematopoietic stem cells have been isolated and have been shown to proliferate and differentiate into myelomonocytic cells, B cells and T cells. (See "Purification and Characterization of Mouse Hematopoietic Stem Cells," Spangrude, Heimfeld and Weissman, Science, vol. 241, pp. 58-62, July 1, 1988.) Research on these cells indicates that the growth of blood forming stem cells and the triggering of their differentiated progeny can be orchestrated by the manipulation of microenvironmental factors known as "growth factors." Building on this knowledge, SyStemix is concentrating on the study of both pluripotent and lineage-restricted human stem cells.

SCID-hu mice represent the only current means by which human hematopoietic stem cells can be identified and isolated. Serving as bioassay systems, they are enabling the company to identify stem cells *in vivo* and study the orderly progression of the human hematopoietic lineages. Over time, SyStemix expects to identify, purify and characterize individual types of stem cells obtained from either donated fetal tissue or from adult bone marrow. Simultaneously, the growth factors necessary for their proliferation can also be identified, purified and produced through the use of standard protein chemistry and recombinant DNA technologies. The com-

pany's ultimate goal is the utilization of appropriate growth factors — to manipulate and proliferate specific kinds of stem cells in a manufacturing environment, and to provide these cells for use in bone marrow transplantation.

While this is a long-term research project at SyStemix, the potential is significant, particularly for victims of cancer, radiation, toxic substances and genetic defects that damage or destroy blood cells. Used alone or in tandem, purified stem cells and their associated growth factors could provide a number of benefits. For example:

- Current bone marrow transplant procedures could become safer, more efficient and far more successful.
 - Instead of trying to work with the vast array of differentiated stem cells, physicians would be able to obtain from SyStemix the pluripotent stem cell that can completely reconstitute all blood elements.
 - Graft-versus-host disease would be eliminated, since the highly purified cells would not contain T cells that distinguish between tissue that is foreign, or "non-self," and tissue that belongs in the body, "self."
- The potential for bone marrow transplantation (BMT) could be significantly increased.
 - The higher rate of success for BMT would allow physicians to utilize this procedure earlier and more often as a treatment procedure.
 - Additional diseases could potentially be treated with BMT, particularly solid organ diseases and cancers of the immune system.
 - Lineage-restricted stem cells could be provided to patients who lack certain blood elements (as in cases of lymphopenia, neutropenia, anemia and thrombocytopenia).
 - BMT could provide a vehicle for gene therapy, carrying genes into the body to protect patients from a long list of inborn cell deficiencies and inborn errors of metabolism.
- The success rate for solid organ transplants would rise with improvements in BMT.
 - Prior to organ transplantation, recipients could receive BMT to prevent the histocompatibility complications that so often threaten transplant procedures today.

**David Rubinfien,
President and Chief Executive Officer**

Highly experienced in building and managing innovative companies, Mr. Rubinfien has had an illustrious career in both the United States and Japan. He was most recently Chairman and Chief Executive Officer of Microgenics Corporation, a privately-held California company utilizing recombinant DNA technology to custom-engineer health care products. He was also a Vice President of Syntex Corporation and President of that company's Syva Corporation subsidiary. He helped to form Syntex Diagnostics Division, and also helped to create Genetic Systems Corporation and Oncogene Corporation. In addition, Mr. Rubinfien was Executive Vice President of Neva Corporation (now Anelva) in Tokyo, and held various other management and scientific positions in Japan and the United States. He holds an M.S. in mathematics and physics from Illinois Institute of Technology, and an M.B.A. from the University of Chicago.

**Joseph McCrary (Mike) McCune III, M.D., Ph.D.,
Scientific Director and co-founder of SyStemix**

Inventor of the SCID-hu mouse model, Dr. McCune has focused his career on understanding the human immune system. In addition to his duties at SyStemix, he is a practicing physician in the AIDS Clinic at San Francisco General Hospital. Dr. McCune conducted his postdoctoral work in the Department of Pathology with Irving Weissman at Stanford University School of Medicine, where he concentrated on pathogenic mechanisms of HIV and on development of the SCID-hu mouse. Previous research experience includes: the immunochemistry of human erythrocyte alloantigens with Georg Springer at Northwestern University; the immunochemistry of human major histocompatibility antigens with Jack Strominger at Harvard University; and the biogenesis of membrane-bound and secreted immunoglobulins with Henry Kunkel and Gunter Blobel at Rockefeller University. Dr. McCune received his M.D. from Cornell University Medical College, and his Ph.D. from Rockefeller University. Board Certified in internal medicine, he completed his residency at the University of California, San Francisco.

**Winnie H. Wan, Ph.D.,
Vice President**

Dr. Wan has a diverse business background with companies engaged in cell transplantation, cell biology and blood products. She was previously at Hana Biologics, where she served as Director of Marketing for diagnostic products, Director of Marketing for cell biology products, and Product Manager for cell biology. Prior to her work at Hana, Dr. Wan was Manager of Financial Planning at the Hyland Therapeutic Division of Travenol Laboratories, and also Product Manager of therapeutic immunoglobulin products worldwide. Prior to entering the business world, Dr. Wan conducted post-doctoral research in the Department of Chemical Biology at Rockefeller University. She received her Ph.D. in biochemistry from Yale University, and an M.B.A. from Columbia University.

**Bertram I. Rowland, J.D., Ph.D.,
General Counsel**

Unusually experienced in patent matters related to biotechnology and other areas of the life sciences, Dr. Rowland was most recently a senior partner at the law firm of Leydig, Voit & Mayer. Among his accomplishments, Dr. Rowland was the attorney for the Cohen-Boyer Stanford University and University of California patent on chimeric plasmids; developer of the patent portfolio of Syva Corporation (a subsidiary of Syntex Corporation); attorney for In re Lundak, which determined the latest rules concerning deposits for microorganisms; as well as attorney for many other patents on behalf of Stanford University, the University of California, and a number of start-up companies engaged in molecular biology, chemistry, immunology and immunodiagnostics. Dr. Rowland received his Ph.D. in organic chemistry from the University of California, Los Angeles, and his J.D. law degree from George Washington University.

Irving L. Weissman, M.D.

Co-founder of SyStemix;

Professor, Department of Pathology, Stanford University;

Investigator, Howard Hughes Medical Institute.

Dr. Weissman brings to SyStemix considerable expertise in hemato-lymphoid differentiation, molecular biology of lymphocyte surface receptors, viral oncogenesis and cellular immunology. With nearly 300 publications to his credit, he is widely recognized for his leadership in studying various aspects of the immune system and, most recently, hematopoietic stem cells. He received his M.D. from Stanford University.

David Baltimore, Ph.D.,

Co-founder of SyStemix;

Professor of Biology, Massachusetts Institute of Technology;

Director of the Whitehead Institute for Biomedical Research.

Awarded the Nobel Prize in Physiology/Medicine for his discovery of reverse transcriptase and granted many other honors for his medical research, Dr. Baltimore is an exceptionally prolific scientist. An active contributor to company research decisions, he brings to SyStemix a wealth of experience in virology, retrovirology and other areas of interest to the company. Dr. Baltimore received his Ph.D. from Rockefeller University.

Leroy E. Hood, M.D., Ph.D.,

Co-founder of SyStemix;

**Bowles Professor of Biology, Chairman of the Division of Biology, and
Director of the Cancer Center, California Institute of Technology.**

Dr. Hood has extensive expertise in genetics, antibodies, MHC genes, protein evolution, protein chemistry, immunochemistry, and micro-chemical instrumentation. His long list of honors includes the the Albert Lasker Basic Medical Research Award for studies of immune diversity, 1987, and the Dickson Prize in Medicine for contributions to immunology and molecular biology, 1987-88. Dr. Hood received his M.D. from the Johns Hopkins School of Medicine, and his Ph.D. from the California Institute of Technology in immunoglobulins: structure, genetics and evolution.

Stanley Falkow, Ph.D.,
Professor of Microbiology and Immunology, and
Professor of Medicine, Stanford University School of Medicine.

Researching the molecular basis of infectious diseases, Dr. Falkow has published widely on the genetic analysis of disease-causing organisms and on cellular invasion by bacteria and other microbes. Among his many responsibilities, he served four years as a member of the Board of Scientific Counselors at the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health. Dr. Falkow received his Ph.D. in biology from Brown University.

Philippa Marrack, Ph.D.,
Professor, Departments of Biophysics, Biochemistry, Genetics, and
Microbiology and Immunology,
University of Colorado Health Sciences Center;
Investigator, Howard Hughes Medical Institute;
Head of the Division of Basic Immunology,
National Jewish Center for Immunology and Respiratory Medicine.

Focusing on research aimed at understanding the immune system, Dr. Marrack has published extensively on the role of various immune proteins and their receptors, with particular emphasis on T cell responses to antigens and the major histocompatibility complex. She received her Ph.D. in biological sciences from New Hall, Cambridge, England.

Owen N. Witte, M.D.,
Professor, Department of Microbiology,
University of California, Los Angeles;
Investigator, Howard Hughes Medical Institute.

Dr. Witte's research interests center on the development of the immune response and growth regulation of hematopoietic stem cells by the ab1 oncogene and other mechanisms. Among his more recent published projects: viral and cellular forms of the Abelson (ab1) oncogene; critical techniques for the study of the immune system; the molecular pathogenesis of Ph-positive leukemias; analysis of B lymphocyte lineages and diversity using long-term lymphoid cultures. Dr. Witte received his M.D. in molecular virology, immunology and medicine from Stanford University School of Medicine.

In the area of SCID-hu improvement:

**Henry C. Outzen, Ph.D.,
Director, Animal Production**

Previously Director of the Prehn Laboratory at the Institute for Medical Research, Dr. Outzen has a strong background working with research-grade mice and murine-associated products (including the production of monoclonal antibodies, the collection of murine tissues and fluids, and surgical organ ablations). He was formerly a scientist at the Jackson Laboratory, conducting research funded by the National Institutes of Health. Dr. Outzen received his Ph.D. in anatomy at the University of Utah College of Medicine.

Specializing in human T lymphotropic viruses and infectious diseases:

Hideto Kaneshima, M.D., Ph.D.

A board certified pathologist from the faculty of Nagoya University in Japan, Dr. Kaneshima is one of the developers of the SCID-hu mouse and one of the first scientists to show that this animal model permits HIV infection. His previous research focused on the biology of the thymus, and the contribution of the thymic stroma to thymocyte differentiation. Dr. Kaneshima graduated with an M.D. and a Ph.D. from Nagoya University School of Medicine.

Reiko Namikawa, M.D., Ph.D.

Dr. Namikawa is a board certified pathologist from the faculty of Aichi Medical University in Japan. One of the developers of the SCID-hu mouse, she created the techniques used to demonstrate HIV infection in the animal model. She also has contributed many advances to the understanding of normal human thymus ontogeny. Dr. Namikawa received both her M.D. and Ph.D. from Nagoya University School of Medicine.

Chu-Chih Shih, Ph.D.

A retrovirologist, Dr. Shih has contributed great insight into the specificity of retroviral integration in the mammalian genome. He was previously in the laboratory of John Coffin at Tufts University, where he received his Ph.D.

In the area of human stem cells and growth factors:

Shelly Heimfeld, Ph.D.

Dr. Heimfeld is one of the Stanford scientists who initially isolated and characterized the mouse hematopoietic stem cell. His Ph.D. research was directed towards an understanding of the developmental biology of another eukaryotic organism (hydra), while working with Hans Bode at the University of California, Irvine.

Ann Tsukamoto, Ph.D.

A scientist with extensive experience in immunology, virology and oncogenesis, Dr. Tsukamoto demonstrated the relation of the int-1 gene to mammary gland hyperplasia while in the laboratory of Harold Varmus at the University of California, San Francisco. She received her Ph.D. from the University of California, Los Angeles.

Yukoh Aihara, M.D., Ph.D.

Board certified in pediatric medicine, Dr. Aihara was previously a faculty member at Yokohama University in Japan. While working with Jan Klein at the Max-Planck-Institut in Germany, he discovered the antibody against Sca-1 that led to isolation of the mouse hematopoietic stem cell. Dr. Aihara received his M.D. and Ph.D. degrees from the Yokohama City School of Medicine.

Chuck Baum, M.D., Ph.D.

A pathology resident at Stanford University and recipient of a fellowship from the Arthritis Foundation, Dr. Baum has had extensive training in B cell development, while completing his Ph.D. studies in the laboratory of Joseph Davie at Washington University, St. Louis.

In the area of T and B cell function:

Richard Harmon, Ph.D.

Dr. Harmon has a depth of experience working with monoclonal antibody technology and many areas of T and B cell function. He was most recently Director of Immunology at XOMA Corporation, and formerly the Manager of Monoclonal Antibody Research and Development at the Cutter Group,

Miles Laboratories. Dr. Harmon received his Ph.D. in immunology and immunogenetics from the University of California, Los Angeles, School of Medicine.

Phillip Streeter, Ph.D.

Dr. Streeter conducted his post-graduate work at Stanford University, in the area of lymphocyte homing. He is the discoverer of "vascular addressins," endothelial cell-surface receptors which direct movement of lymphocytes into lymphoid organs. Dr. Streeter has concentrated on the mechanisms which lead to the movement of lymphocytes into areas of chronic inflammation. He received his Ph.D. from Kansas State University in tumor immunology.

John Krowka, Ph.D.

Dr. Krowka has a background in the processes of normal mouse cytotoxic T cells. He was formerly in the laboratory of Dan Stites at the University of California, San Francisco, where he devoted his research efforts to studying the particular problem of T cell function and dysfunction during HIV infection in man. Dr. Krowka received his Ph.D. in immunology from the University of Alberta.

Additional research team:

In addition to the above-mentioned Ph.D. and M.D. scientists, SyStemix is building a strong support group of researchers with M.S. and B.S. degrees. They, too, contribute unique skills in areas of research related to the company's goals.

SyStemix is very proud of its research team and believes this highly talented group will provide important insight into disease processes and treatment responses. The company looks forward to working in partnership with other organizations, as well as independently, to commercialize a range of new products to finally overcome difficult-to-treat diseases involving the human immune system.