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Clinical-Scale Production of Antigen-Specific T Cells Directed Against Hepatitis B Virus

BY ALAN HARDWICK, DAVID MCMILLEN, JENNIFER MARTINEZ, AMANDA AUSTIN, AARON POSEY, CONNIE AVE-TEEL, PHILLIP MAPLES, and SUSANNE SCHNEIDER

A clinical-scale manufacturing process has been developed for the *ex vivo* expansion of autologous cytolytic T lymphocytes (CTLs) directed against cells infected with the hepatitis B virus (HBV). The process is based on the Rapid Expansion Method (REM) technology originally developed at the Fred Hutchinson Cancer Research Center in Seattle, WA, by Greenberg and Riddell.[1,2]

Preparations are underway to initiate a company-sponsored Phase I clinical trial in which REM will be used to expand rare autologous HBV-specific CTLs that will then be infused to patients chronically infected with HBV. Earlier studies have shown that such patients mount only a weak CTL response to HBV.[3-5] Chronic hepatitis B can lead to severe liver damage such as cirrhosis and hepatocellular carcinoma.[6] By infusing clinical-scale quantities of autologous HBV-specific CTLs into chronic HBV patients, it may be possible to boost the immune system so that it can control the viral infection.[7]

As part of the clinical trial preparation, a series of full-scale experiments was carried out to qualify the REM process and the manufacturing parameters. This process qualification study consisted of experiments in which 100 ml of blood were obtained from seven chronic HBV patients. Rare autologous HBV-specific CTLs were isolated from this blood and then REM-expanded to form a bank of cryopreserved product intermediates (CPIs). These CPIs were tested extensively to ensure the purity, viability, functionality, and specificity of the HBV-specific CTLs in each patient-specific CPI aliquot. Each CPI aliquot was then thawed and REM-expanded in a medical grade plastic bag to generate 2-3 x 10^9 sterile, patient-specific, HBV-specific CTLs.

Materials and Methods

Preparation of Cells from Patients

100 ml of whole blood was obtained from each of seven patients chronically infected with HBV. The blood was centrifuged over Ficoll® (Pharmacia) and the resulting layer of peripheral blood mononuclear cells (PBMCs) was collected and split into four aliquots:

- Approximately 100 x 10^6 autologous PBMCs were mixed with anti-CD8 antibody-coated magnetic microparticles (BioTransplant, Medford, MA) to select $CD8^+$ cells. Cells bound to the microparticles were washed and then released from the particles by mechanical agitation. (Fraction 1)
- Approximately 10 x 10^6 autologous PBMCs were allocated for use as filler cells. (Fraction 2)
- 2-3 x 10^6 autologous PBMCs were allocated to serve as antigen-presenting cells. These PBMCs were incubated overnight at 37°C with GMP recombinant vaccinia viral vector containing the DNA sequence for HBV core protein (Novavax, Rockville, MD). (Fraction 3)
- Approximately 20 x 10^6 autologous PBMCs were allocated for later use as target cells in a ^{51}Chromium-release assay. These PBMCs were put into culture with B95-8 virus and cyclosporin A to generate autologous Epstein-Barr virus-transformed B lymphoblastoid cell lines (EBV-LCLs).[8]

Preparation of Allogeneic Filler Cells

REM expansion of T cells requires the use of two types of "filler cells" as process reagents. These are PBMCs (from a leukapheresis unit) and EBV-LCLs (from a cryopreserved bank).

PBMC Filler Cells. Apheresed PBMCs were obtained from an unmatched donor. Donors were tested for hepatitis B, hepatitis C, HIV, and syphilis (standard screening for all

Alan Hardwick, Ph.D. *(hardwick@targen.com) is vice president of process development, David McMillen is associate director of process development, Jennifer Martinez is a research assistant, Amanda Austin is a research associate, Aaron Posey is a research assistant, Connie Ave-Teel is a research group leader, Phillip Maples, Ph.D. is vice-president of technical operations, and Susanne Schneider, Ph.D. is director of research at CellExSys,Inc., Seattle, WA.*

Table 1: Results of the First REM Expansion (in 96-well plates)

T cells/well	Number of HBV Patients Processed	Number of Growth Positive Wells per HBV Patient	
		Average	Std. Dev.
1	7	344	179
5	7	322	180

Table 1 shows that for each HBV patient the 40 plates seeded with 1 T cell/well gave an average of 344 "growth positive" wells, which corresponds to a plating efficiency of 9%. The 10 plates seeded with 5 T cells/well gave an average of 322 "growth positive" wells, which corresponds to a plating efficiency of 33.5%.

Table 2: Results of the Second REM Expansion (in 24-well plates)

HBV Patient Number	Number of Wells of HBV Core-Specific CTL	Number of Viable Cells at End of Expansion (x 10^6)	Number of Wells of HBV Core-Specific CTL chosen for Further Expansion
1	2	8.2 ± 0.5	2
2	1	4.0	1
3	1	2.3	1
4	8	3.6 ± 3.5	6
5	7	3.4 ± 2.3	7
6	5	5.2 ± 1.3	5
7	10	5.1 ± 1.7	10
Totals: N=7	34	4.5 ± 2.5 (Avg)	32

Table 2 shows that 34 HBV core-specific CTL isolates were obtained from the seven chronic HBV patients. These 34 CTL isolates contained an average of 4.5 x 10^6 cells after the second REM expansion. Two of the 34 CTL isolates contained less than 0.5 x 10^6 cells after the second REM expansion and therefore these two isolates were not processed further.

blood donors in the United States). The PBMC were divided into two portions. The first portion was centrifuged over Ficoll to remove the red blood cells and platelets, gamma-irradiated (3,500 rads), and used as fresh PBMC filler cells in the first REM expansion. The remaining PBMCs from the apheresis unit were washed using a Cobe® 2991 cell processor, then cryopreserved and stored in liquid nitrogen. These PBMCs were thawed, washed, and gamma-irradiated (3,500 rads) when needed as filler cells in subsequent REM expansions. For all REM expansions, T cells from a given HBV patient were exposed to PBMC filler cells from a single apheresis donor.

EBV-LCL Filler Cells. An aliquot of allogeneic EBV-LCLs was thawed from the working cell bank and placed in culture. These cells were passaged as needed. The cultured EBV-LCLs were harvested and gamma-irradiated (10,000 rads) when needed as filler cells in REM expansion.

Preparation of Cryopreserved Product Intermediates

To enrich the rare HBV-specific CTLs in the autologous CD8-selected cells (Fraction 1), these cells were co-cultured with the autologous PBMC filler cells (Fraction 2) and the autologous antigen-presenting cells (Fraction 3). Prior to this *in vitro* stimulation step, the autologous PBMC filler cells were gamma-irradiated (3,500 rads) to prevent them from expanding and the autologous antigen-presenting cells were UV-irradiated to inactivate the vaccinia virus. These three cell fractions were co-cultured for 14 days at 37° C, 5% CO_2 in complete media. Complete media consisted of RPMI 1640 media supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 0.005 µM 2-mercaptoethanol.

After 14 days of *in vitro* stimulation, the cultured cells were harvested and put into limiting dilution cultures. That is, the cells were placed in 96-well plates at T cell/well ratios of 1 and 5. Each well also contained gamma-irradiated (3,500 rads) allogeneic PBMC filler cells, gamma-irradiated (10,000 rads) allogeneic EBV-LCL filler cells, the monoclonal antibody OKT3 (10 ng/ml, Orthoclone®), and IL-2 (25 U/ml, Chiron) in complete media. The plates were then incubated for 14 days at 37° C, 5% CO_2.

After 12 to 14 days in culture, the 96-well plates were scored for "growth positive" wells. These wells were harvested and transferred to a 24-well plate for further expansion. Each well also contained thawed gamma-irradiated (3,500 rads) allogeneic PBMC filler cells, passaged gamma-irradiated (10,000 rads) allogeneic EBV-LCL filler cells, and OKT3 (10 ng/ml) in complete media and IL-2 (25 U/ml). The 24-well plates were then incubated for 14 days at 37° C, 5% CO_2. The cultures were fed with fresh media and IL-2 on day 4 or 5, day 7 or 8, and day 10 or 11.

After 11 to 13 days in culture in the 24-well plates, cells from each well were assayed by a ^{51}Chromium-release assay for specific cytolytic activity against HBV core-expressing target cells that consisted of autologous EBV-LCLs. The T cells that showed specific cytolytic activity against HBV core-expressing

target cells were harvested and put into a gas permeable culture bag (Baxter Lifecell®) for further expansion.

Each chosen well of HBV core-specific CTLs was transferred to its own culture bag. Each bag also contained thawed gamma-irradiated (3500 rads) allogeneic PBMC filler cells, passaged gamma-irradiated (10,000 rads) allogeneic EBV-LCL filler cells, and OKT3 (10 ng/ml). The bag cultures were incubated for 14 days in complete media and IL-2 (25 U/ml) at 37° C, 5% CO_2. The bag cultures were fed with fresh media and IL-2 on day 4 or 5, day 7 or 8, and day 10 or 11.

After 11 to 13 days in culture, cells from each bag were again assayed for specific cytolytic activity against autologous HBV core-expressing target cells. Specificity was confirmed by absence of cytolytic activity toward four types of control target cells: uninfected autologous target cells, autologous target cells infected with a vaccinia virus vector control (HBV polymerase), allogeneic target cells derived from the PBMC filler cells, and allogeneic target cells derived from the EBV-LCL filler cells. The cultured cells were also tested on day 14 for cell number, viability, and phenotype. Those CTLs that passed the testing were washed and harvested using a Cobe 2991 device. The harvested CTLs were suspended in a DMSO-based cryopreservative solution, transferred to cryovials (1 x 10^7 CTLs in 1 ml cryosolution per vial), frozen using a controlled rate freezer, and stored in liquid nitrogen. These frozen CTLs were designated as CPIs.

Preparation and Storage of the Final Product

Certain CPIs were chosen to be expanded into a final product. These CPIs were thawed and 5 x 10^6 CTLs were put into culture bags (Lifecell). These cultures were carried out in the same way as the bag cultures described earlier. After 14 days, the bag cultures were washed and harvested using a Cobe 2991 device. The harvested CTLs were formulated in 400 ml of Plasma-Lyte® A/4% human serum albumin (an infusion grade solution) contained in a

Table 3: Acceptance Criteria for CPIs (after the Third REM Expansion)

Test	Acceptance Criteria
Cell Number	≥ 1.0 x 10^9
Cell Viability	≥ 80%
$CD8^+$ Cell Purity	≥ 75%
CTL Specificity for HBV Core	[Specific lysis of HBV core target cells] ≥ 10%. [Specific lysis of control target cells] ≤ 15%. [Max specific lysis of HBV core target cells – Max specific lysis of control target cells] ≥ 10%. Note: E/T Range is 0.01 to 100.

The tests/measurements of Table 3 were performed on samples taken from the respective 32 cultures just prior to harvest. Of the 32 candidates tested, 18 CTL isolates met the acceptance criteria of Table 3. The characteristics of these isolates are shown in Table 4.

Table 4: Results of the Third REM Expansion (in culture bags)

HBV Patient Number	Number of CTL Isolates formed into CPIs	Average Cell Number (x 10^9)	Average Cell Viability	Average Cell Purity	Specificity for HBV Core?
1	2	3.1	97%	96% $CD8^+$ 3% $CD4^+$	Yes
2	0	1.9*	98%*	7%$CD8^+$ 89% $CD4^+$	Yes*
3	1	3.2	96%	97% $CD8^+$ 2% $CD4^+$	Yes
4	2	1.5	97%	81% $CD8^+$ 1% $CD4^+$	Yes
5	5	2.3	87%	98% $CD8^+$ 1% $CD4^+$	Yes
6	1	2.5	96%	98% $CD8^+$ 6% $CD4^+$	Yes
7	7	3.4	97%	99% $CD8^+$ 2% $CD4^+$	Yes
Totals: N=7	18	2.8	94%	96% $CD8^+$ 2% $CD4^+$	Yes

*The candidate CTL isolate from Patient 2 was not formed into a CPI because it did not meet the acceptance criteria of Table 3 for cell purity.

Table 4 shows that 18 HBV core-specific CPI were obtained from the seven chronic HBV patients. These 18 patient-specific CPI lots contained an average of 2.8 x 10^9 cells after the third REM expansion with an average viability of 94% and an average purity of 96% $CD8^+$ cells.

Table 5: Thawing the CPIs in Preparation for Final Expansion

HBV Patient/ CPI Designation	Duration of Storage in LN_2 (Days)	Cell Recovery Post-Thaw	Cell Viability Post-Thaw
Patient 1 #113	33	100%	93%
Patient 1 #241	33	85%	94%
Patient 3 #282	40	79%	98%
Patient 4 #147	19	83%	91%
Patient 5 #467	8	63%	92%
Patient 5 #756	8	77%	99%
Avg (n=6)		86%	95%

Each cryovial contained 10 x 10^6 cells prior to freezing.

Table 6: Results of the Final Expansion and Harvest (Fourth REM Expansion)

HBV Patient/ CPI Designation	Number of Cells at end of Expansion ($x10^9$)	Number of Cells after Harvest ($x10^9$)	Harvest Efficiency	Cell Viability after Harvest
Patient 1 #113	3.1	2.5	81%	93%
Patient 1 #241	3.1	2.7	87%	96%
Patient 3 #282	2.0	2.0	99%	98%
Patient 4 #147	2.3	2.0	88%	98%
Patient 5 #467	2.5	2.0	80%	98%
Patient 5 #756	1.7	1.5	91%	99%
Avg (n=6)	2.5	2.1	87%	97%

600 ml transfer pack (Baxter).

Each respective final product was then stored at room temperature (18-20° C) for 48 hours. Samples were removed for testing after 24 hours and 48 hours in storage.

Results and Discussion

First REM Expansion

After immunomagnetic selection of $CD8^+$ cells and *in vitro* stimulation, autologous T cells from chronic HBV patients were diluted, seeded into 96-well plates, and REM-expanded. Forty plates were seeded with 1 T cell/well and 10 plates were seeded with 5 T cells/well. At the end of the expansion, "growth positive" wells were identified (Table 1).

Second REM Expansion

Starting with 96-well plates seeded with 1 T cell/well, the contents of the "growth positive" wells for each HBV patient were transferred to 24-well plates for further expansion. The maximum number of wells transferred was 500 per patient.

At the end of the REM expansion in 24-well plates, the T cells were tested in a ^{51}Chromium-release assay for their ability to specifically lyse HBV core-expressing target cells. T cells showing cytotoxic specificity were designated as CTLs and were enumerated. Those wells with ≥ 0.5 x 10^6 cells were chosen for further expansion (Table 2).

Third REM Expansion

The thirty-two HBV core-specific CTL isolates from the seven HBV patients were REM-expanded a third time. Each CTL isolate was expanded in its own plastic culture bag. At the end of the expansion, the CTLs were tested for cell number, cell viability, $CD8^+$ cell purity, and specificity (Table 3). The CTLs that met the acceptance criteria were frozen to form CPIs.

Thawing the CPIs

The CPIs were cryopreserved in 1 ml aliquots and stored in a liquid nitrogen tank. After storage for 8 to 40 days, certain CPI aliquots were thawed and prepared for final expansion. Results of the CPI storage/thawing process are shown in Table 5.

Fourth (Final) REM Expansion

Upon thawing the CPI aliquots, 5 x 10^6 thawed cells from each aliquot were placed in a respective culture bag for final expansion. At the end of this 14-day final expansion, the cells were washed and harvested, and placed in their final container (Baxter 600 ml transfer pack) with 400 ml of Plasma-Lyte A/4% HSA. Results of the final expansion and harvest are shown in Table 6.

In addition to testing the final product cells for cell number and viability (Table 6) the final product cells were also tested for $CD8^+$ cell purity and HBV core specificity using the same acceptance criteria as shown in Table 3. For the six tested final products of Table 6, $CD8^+$ cell purity ranged from 95% to 99% and all final products showed specificity for HBV core.

To establish product stability, the six final products were stored at room temperature (18-20° C) for 48 hours after harvest/formulation. Cell viability was 97% ± 2% (Avg ± SD, n=6) after 24 hours and viability was 95% ± 4% (Avg

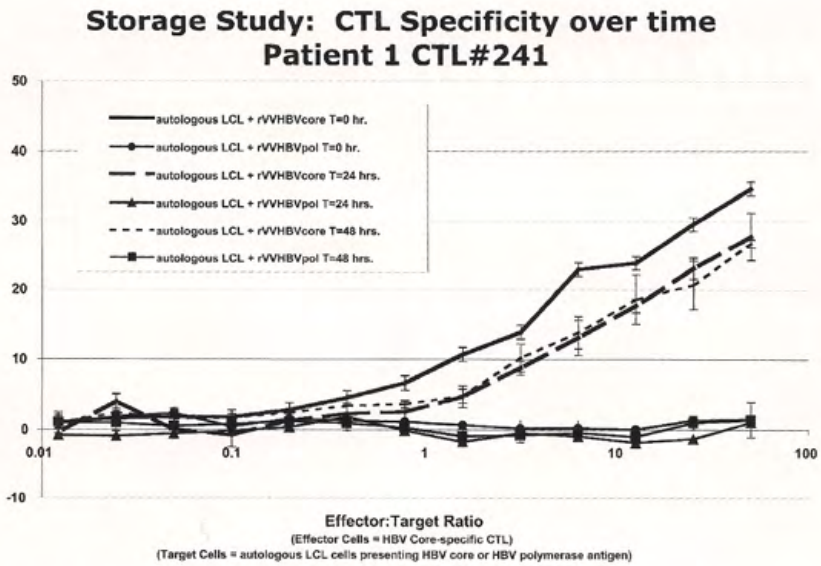

Figure 1: ^{51}Chromium-Release Assay Results for Final Product During Storage

± SD, n=6) after 48 hours of storage. The final product cells also maintained their specificity for the HBV core antigen after 48 hours of storage (Fig. 1).

Conclusion

Starting with 100 ml of blood, the REM process generated sterile, clinical-scale quantities of autologous HBV-specific CTLs for six out of seven chronic HBV patients. For one HBV patient, REM processing yielded only $CD4^+$ CTLs that did not meet specifications for cell phenotype. For the other six chronic HBV patients, REM processing enabled generation of large numbers ($1.5\text{-}2.7 \times 10^9$) of HBV-specific CTLs that met all specifications. During 48-hour storage in an infusion-grade solution at room temperature, the final product CTLs maintained their viability, functionality, and specificity.

The experiments described here served to qualify the *ex vivo* REM process for use in our company-sponsored clinical trial on chronic HBV patients.

REFERENCES

1. Riddell SR, Greenberg PD. The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. *J Immunol Methods* 1990:128;189-201.

2. Greenberg PD, Reusser P, Goodrich JM, Riddell SR. Development of a treatment regimen for human cytomegalovirus (CMV) infection in bone marrow transplantation recipients by adoptive transfer of donor derived CMV-specific T cell clones expanded in vitro. *Ann NY Acad Science* 1991:636;184-195.

3. Rehermann B, Lau D, Hoofnagle JH, Chisari FV. Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. *J Clin Investigations* 1996:97;1655-1665.

4. Rehermann B. Intrahepatic T cells in hepatitis B: Viral control versus liver cell injury. *J Exper Med* 2000:191;1263-1268.

5. Maini MK, Boni C, Lee CK et al. The role of virus-specific $CD8^+$ cells in liver damage and viral control during persistent hepatitis B virus infection. *J Exper Med* 2000:191;1269-1280.

6. The EASL Jury. EASL International Consensus Conference on Hepatitis B. *J Hepatol* 2003:38;533-540.

7. Riddell SR, Geenberg PD. Principles for adoptive T cell therapy of human viral diseases. *Ann Review Immunol* 1995:13;545-586.

8. Sugden B, Mark W. Clonal transformation of adult human leukocytes by Epstein-Barr virus. *J Virol* 1977:23;503-508.