PROGRESS REPORT

RESEARCH PROJECT NUMBER DE-FG07-96ER62331

"A NOVEL BIOMARKER FOR BERYLLIUM SENSITIZATION IN HUMANS"

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Overall, this project is designed to identify the beryllium reactive T-cell clones that are proliferating *in vivo* in individuals sensitized to beryllium. The basic method for identifying such clones is the *hprt* T-cell mutation assay. The rationale is that *in vivo* proliferating T-lymphocytes are more likely to undergo *hprt* mutations and/or be included in *hprt* mutant fractions of T-cells isolated from peripheral blood. T-lymphocytes isolated as *hprt* mutants from beryllium sensitized individuals are propagated *in vitro* and characterized for T-cell receptor (TCR) β gene usage patterns and antigen reactivity. Results will be compared with similar characteristics determined for T-cell clones from the same individuals that were developed *in vitro* from peripheral blood lymphocytes by beryllium stimulation.

This research project has several specific aims. Progress for the year 10/1/96 to 9/30/97 is given by specific aim.

Specific Aim 1: **Identifying those T-lymphocyte subpopulations undergoing *in vivo* clonal amplification in beryllium sensitized individuals using the technique of surrogate selection for somatic mutants.**

Eighteen peripheral blood samples have been obtained from 13 beryllium sensitized individuals, identified and followed by the scientific staff of the Cytogenetics Program at the Oak Ridge Institute for Science and Education. T-cell cloning assays have been performed on 13 of these samples, with the others currently cryopreserved for study in the near future. Wild type and mutant isolates obtained from these cloning assays have been propagated *in vitro* for TCR gene usage pattern and functional characterizations.

The current status of all 18 blood samples and the results of the 13 T-cell cloning assays performed thus far, with the numbers of wild type and mutant isolates derived from each, is given in Table 1. As can be seen from this Table, mutant frequencies in the 13 completed assays range from 0.1 to 87.0 x 10^{-6}. All except the two highest of these (62.5 and 87.0 x 10^{-6} from the same individual blood sample) are within the normal range. The two outliers are probably the result of extremely low cloning efficiencies in the two assays in question.

Specific Aim 2: **Identifying, in many or all such individuals, common TCR β gene and/or junctional region usages in T-lymphocyte clones which have amplified *in vivo.***

To date, 45 wild type and 259 *hprt* mutant T-cell isolates have been obtained from 13 individuals and have been propagated *in vitro* to numbers sufficient for analysis. Many more clones are currently in the process of *in vitro* propagation. Isolated clones reaching sufficient cell numbers are cryopreserved for viability and cell pellets are frozen for DNA characterizations.

Characterizations thus far have been focused on sequence analysis of the TCR β gene variable (BV), junctional (BJ) and CDR3 hypervariable junctional regions. Forty wild type and 180 *hprt* mutant T-cell clones have been sequenced to date. Sufficient mutant isolates have been characterized for two individuals to allow preliminary identification of potential usage patterns (Tables 2-5). As can be seen, in one individual (AB), a large amplifying *in vivo* clone has been identified in two blood samples obtained approximately five months apart. In the other individual (ES), a smaller amplifying clone was observed in only one blood sample. Of significance, one identical BV 2, BJ 2s5 expressing T-cell clone was identified in both individuals at different times. Specific amplifying T-cell clones are potential candidates for beryllium reactive T-cells. The isolates with identical TCR β gene expression found in two individuals are strong candidates for being representatives of an *in vivo* beryllium reactive clone.

Specific Aim 3: **Determining specific reactivity of the *in vivo* amplifying T-lymphocyte clones when challenged with beryllium and appropriate control antigens.**

Before functional analyses for antigen reactivity can be accomplished, a source of HLA identical antigen presenting cells must be obtained from each individual. We initially planned to use autologous peripheral blood lymphocytes for this purpose but quickly found that this would be too expensive of T-cells. Therefore, B-lymphoblastoid cell lines have had to be established for each individual to use as antigen presenting cells in beryllium stimulation assays.

Thus far, EBV lines have been established to develop a continuing source of antigen presenting cells for six subjects. Transformations are in progress and will be accomplished for the remaining subjects with samples in our possession over the next several weeks.

We have selected two *hprt* mutant T-cell isolates at random from the cloning assays thus far performed. These isolates have been stimulated with autologous and heterologous EBV transformed cell lines in the presence of beryllium. Neither isolate appeared to represent a large *in vivo* clone as judged by the finding of many isolates expressing the identical TCR gene sequence. The beryllium stimulations were begun only ten days ago and it is unclear at present whether any beryllium response is occurring. We plan to test for response by measuring IL-2 levels in the supernatant.

The most interesting clone to be studied in this regard comes from subject AB as noted in the Tables. This large *in vivo* clone is represented by eight TCR gene identical mutant isolates derived from two independent blood samples. This suggests a very large clone which, in a beryllium

sensitized individual, may have arisen through beryllium stimulation. We are currently awaiting a repeat blood sample from this subject to establish an EBV transformed B-lymphoblastoid line as the source of antigen presenting cells so that beryllium responsivity of this clone can be assessed.

Specific Aim 4: Generating *in vitro* and isolating beryllium reactive T-lymphocyte clones from peripheral blood lymphocytes obtained from the same beryllium sensitized individuals.

Attempts to develop T-cell lines directly from peripheral blood samples from subjects immediately on receipt of the sample from Oak Ridge has been attempted on three occasions without success. However, after the stimulated cultures had been established by us, we learned that the blood samples did not show positive results on the beryllium lymphocyte proliferation test assay performed in Oak Ridge, even though in the past the subject had responded vigorously. Therefore, it is possible that in these three samples, beryllium reactive cells were not present in the peripheral blood at the time we received them. In the future, we will test samples in Vermont for beryllium proliferative capacity prior to setting up the long term cell lines as the beryllium response *in vitro* is capricious. We had hoped to conserve time and blood cells by avoiding this step but it appears to be necessary.

Specific Aims 5, 6 and 7 involve identifying common TCR β gene BV, BJ, and junctional region usages in the *in vitro* derived beryllium reactive T-cell clones, comparing TCR BV, BJ and junctional region usages between the *in vivo* and the *in vitro* derived clones to identify further commonality and to develop a quantitative polymerase chain reaction (qPCR) method for amplifying common (and therefore relevant) specific TCR β genes directly from the peripheral blood of sensitized individuals. Work on these specific aims is planned for the later phases of this project as this work will be based on the results obtained from the studies underlying specific aims 1 through 4.

Work for the second year (10/l/97 - 9/30/98) will continue in the main as originally proposed. However, two changes in methodology are currently being contemplated.

1. Mass T-cell cultures grown in thioguanine, followed by qPCR of TCR cDNAs using BV and BJ specific primers may be tried to speed the process of TCR gene usage identification. If this method is employed, the PCR TCR cDNA products will then be cloned in Bluescript phagemids for sequence analyses.

2. Beryllium may be employed in place of PHA for some of the *hprt* T-cell cloning assays on individuals with extremely high beryllium lymphocyte proliferation test results. Using beryllium in place of PHA will, in effect, preselect for beryllium reactivity among the *in vivo* proliferating T-cell clones.

TABLE1

					Isolates	
Date Received	Expt. #	Subject	Cloning Efficiency	Mutant Frequency	Wild Type	Mutants
12/3/96	LS 909	GF	0.07	11.8 (x 10^{-6})	9	11
2/28/97	LS 913	AB	0.20	10.0	11	11
3/4/97	LS 914A	JH	0.007	62.5	-	-
3/4/97	LS 914B	ES	0.18	5.8	3	12
3/4/97	LS 926	JH	0.006	87.0	-	-
7/17/97	LS 928	GF	0.12	10.1	1	9
7/29/97	LS 931	AB	0.14	8.8	4	7
7/30/97	LS 932	JH	0.03	5.3	3	2
9/10/97	LS 934A	ER	1.7	0.9	1	44
9/10/97	LS 934B	GF	0.31	3.7	1	7
9/19/97	LS 935A	JK	0.27	9.1	4	18
9/19/97	LS 935B	JL	0.17	15.9	3	47
9/19/97	LS 935C	RZ	0.15	19.0	1	65
10/8/97	LS 938A*	SF				
10/8/97	LS 938B	FM				
10/8/97	LS 938C	KB				
10/15/97	LS 938D	FA				
10/15/97	LS 938E	KM				

* Peripheral blood mononuclear cell samples for expt. #s LS 938A→E currently cryopreserved.

TABLE 2

TCR β GENE USAGE
Beryllium Subject: ES

Isolate #	BV #[3]	BV Sequence[4]	CDR 3[5]	BJ Sequence[6]	BJ #[7]
		Experiment LS 914B - 3/4/97			
WT2[1]	4	CS	VGGTI	YEQYFG	2s7
WT7	2s1	CSA	SAPRRGT	YEQYFG	2s7
WT9	14	CASS	IGQVP	NSPLHFG	1s6
WT14	13s2	CAS[8]	RDSGSF	YEQYFG	2s7
M1[2]	3	CASS	MGTAYRI	DGYTFG	1s2
M7	3	CASS	MGTAYRI	DGYTFG	1s2
M8	3	CASS	MGTAYRI	DGYTFG	1s2
M2	14	CASS CASS	PGVAF - - - - -	SPLHFG - - - - -	1s6 1s2 (FS)
M3	9	CASS	FTVSM	YFG	2s5
M4	2s1	CSA	MSGKS	NFQFFG	2s1
M5	7	CASS	QDKWGQGLRA	EAFFG	1s1
M12	21	CASS	QSGLG	GYTFG	1s1
M14	13s2	CAS	RDSGF	YEQYFG	2s7
M16	21	CASS	LDHYRGS	DTQYFG	2s3
M22	6	CASS	DGGGRAI	EGFFG	2s1

(1) WT = Wild type isolate
(2) M = Mutant isolate
(3) BV # = TCR β variable gene family (subfamily)
(4) BV Sequence = Amino acid (single letter code) of BV near the hypervariable junctional region
(5) CDR 3 = Complementarity Determining Region 3 = Amino acid sequence of hypervariable haptene binding region of TCR β gene
(6) BJ Sequence = Amino acid sequences of BJ near the hypervariable junctional region
(7) BJ # = TCR β junctrional gene family (subfamily)
(8) Highlights indicate within person homologies

TABLE3

TCR β GENE USAGE
Beryllium Subject: ES

Isolate #	BV #[3]	BV Sequence[4]	CDR 3[5]	BJ Sequence[6]	BJ #[7]
		Experiment LS 927 - 7/1697			
M18[9][2]	2	CSA	RRVPAS	QETQYFG	2s5
M1B	9	CASS	RTSGTG	NEQFFG	2s1
M3B	9	CASS	QDESEGSA	EAFFG	1s1
M3A	7	CASS	QAAEL	DTQFFG	2s3
M25	9	CASS	LGLAGLGD	EQYFG	2s7
M12	5	CASS	LVEKGS	SYNEQFFG	2s1
M27	6	CASS	AATSGA	PYEQYFG	2s7
M30	4	CS	VEASIAGGRY	EQFFG	2s1
M10	12	CA	IMRGQGGE	TGYFG	2s5
M7B	7	CASS	KHDLGRGYTFG		
M21	1	CASS	ELAGGH	EQFFG	2s1
M16	1	CASS	ADGTRET	QYFG	2s5
M11	2	CSA	KDPSRG	YEQYFG	2s7
M8	2	CSA	RPSKSET	QYFG	2s5
M33A	13	CASS	TSGRDD	TQYFG	2s3
M33B	10	CA			2s2
M17					1s3
M26	7	CASS	QKP	GGSNQPQHFG	1s5
WT13[1]	2	CSA	RSG	SYNEQFFG	2s1

Legend same as for Table 2

[9] Highlight indicates between person homology

TABLE 4

TCR β GENE USAGE
Beryllium Subject: AB

Isolate #	BV #[3]	BV Sequence[4]	CDR 3[5]	BJ Sequence[6]	BJ #[7]
		Experiment LS 913 - 2/28/97			
WT2[1]	8	-	-	-	2s3
WT3	5	CASS	LAAPR	GEKLFFG	1s4
WT4	3	CAS	TEHANT	GELFFG	2s2
WT5	2s1	CSA	RSGDL	YNEQFFG	2s1
WT6	22/23 6	CASS CASS	EAPIMOA PTVASGGP	DTQYFG SQKHSVLRR	2s3 2s4 (FS)
WT8	14	CASS	FGTGVV	GELFFG	2s2
WT9	17	CASS	IPRGW	QFFG	2s1
WT10	7s2	CASS	QDGSPG	DTGELFFG	2s2
WT11	6	CASS	LDWDI	QETQYFG	2s5
WT12	2s1	CSA	PLELLRV[8]	SYNEQFFG	2s1
WT17	2s1	CSA	PLELLRV	SYNEQFFG	2s1
M1[2]	13s2	CASS	YGAE	SGNTIYFG	1s3
M4	13s2 2	CASS CS	YGAE GRSGDIY	SGNTIYFG NEQFFG	1s3 2s1
M6	13s2	CASS	YGAE	SGNTIYFG	1s3
M3	14	CASS	YGA	NEQFFG	2s1
M2	13s2	CASS	RGHVGRD	SPLHFG	1s6
M9	12s2	CAIS	STSGNT	YNEQFFG	2s1
M10	12s2	CAIS	STSGNT	YNEQFFG	2s1
M13	12s2	CAIS	STSGNT	YNEQ|LLLRA	2s1
M5[9]	2	CSA	PRRWPAS	QETQYFG	2s5
M18	21	CASS	LVSARD	TGELFFG	2s2
M19	3	CASS	FFGNRGP	NTEAFFG	1s1

Legend same as for Tables 2 and 3

TABLE 5

TCR β GENE USAGE
Beryllium Subject: AB

Isolate #	BV #[3]	BV Sequence[4]	CDR 3[5]	BJ Sequence[6]	BJ #[7]
		Experiment LS 931 - 7/2997			
WT4[1]	5	CASS	LEVSGH	[8]SGNTIYFG	1s3
M10A[2]	13	CASS	YGAE	SGNTIYFG	1s3
M13	13	CASS	YGAE	SGNTIYFG	1s3
M2	13	CASS	YGAE	SGNTIYFG	1s3
M8B	13	CASS	YGAE	SGNTIYFG	1s3
M5A	13	CASS	YGAE	SGNTIYFG	1s3

Legend same as for Tables 2 and 3