Brief Definitive Report

HERPESVIRUS SAIMIRI STRAIN 11 IMMORTALIZES A RESTRICTED MARMOSET T8 LYMPHOCYTE SUBPOPULATION IN VITRO

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Some viruses preferentially infect restricted subpopulations of lymphocytes. The pathogenesis of the diseases induced by these lymphotropic viruses is determined at least in part by this selectivity of infection. *Herpesvirus saimiri*, a lymphotropic herpesvirus that causes no apparent disease in its natural host *Saimiri sciureus* (squirrel monkey), induces a fulminant lymphoproliferative syndrome in a variety of other New World primate species (1). Animals die after experimental *H. saimiri* infection, with manifestations that may include leukemia, parenchymal lymphocytic infiltration, and occasionally solid tumor masses. Lymphoblastoid cell lines derived from these tumors have been shown to express a number of T cell–associated membrane structures (2). While extensive characterization of this virally induced disease has been carried out, little work has been done to elucidate the nature of the interactions between *H. saimiri* and the T lymphocyte.

We have recently shown (3) that PBL of *Callithrix jacchus* (common marmoset) can be reproducibly immortalized in vitro by *H. saimiri*. This has facilitated the examination of interactions between this lymphotropic virus and its target cells. In the present study we have shown that lymphoid lines derived from common marmoset PBL by in vitro immortalization with *H. saimiri* strain 11 have NK cell function and phenotypic markers of both T cells (T12, T8) and NK cells (NKH1). In fact, the *H. saimiri* strain 11 immortalized cell represents a remarkably restricted subset of the PBL population of the common marmoset.

Materials and Methods

Cell Lines. Lymphoid cell lines were established by immortalization of common marmoset PBL with cell-free *H. saimiri* strain 11 grown in owl monkey kidney cells, as previously described (3). The lymphoid cell lines were maintained in vitro from 3 mo–1 yr.

NK assay. Cytotoxicity assays were performed in V-bottomed microtiter plates (Linbro Chemical Co., Hamden, CT) using 5,000 ^{51}Cr-labeled target cells per well in a total volume of 150 μl RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Sterile Systems, Inc., Logan, UT). After a 6 h incubation of effector cells with target cells, aliquots of supernatants from each well were counted in a

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gamma counter. Percent specific release was calculated by the formula [(test release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Phenotypic Analyses. The mAbs used in these studies included anti-T12 (pan–T cell), anti-T11 (sheep erythrocyte rosette receptor), anti-T8 (21Thy2D3, suppressor/cytotoxic), anti-NKH1$_A$ (NK-associated, μ isotype), anti-NKH1 (NK-associated, γ1 isotype), and anti-NKH2 (NK-associated). These antibodies were provided by S. Schlossman, Dana-Farber Cancer Institute, Boston, MA. Anti-T4 (94b1, helper/inducer) was provided by R. Winchester, New York University School of Medicine, New York. These mAbs were selected for their reactivity with conserved determinants expressed on lymphocytes of the common marmoset (4). Anti-NKH1$_A$ and anti-NKH1 define an NK cell–associated antigen expressed on the majority of human NK cells, whereas anti-NKH2 reacts with a subset of large granular lymphocytes (LGL) in human PBL that make up only a portion of cells containing NK activity (5, 6).

In vitro–immortalized cells were stained with mAbs and analyzed by flow cytometry as previously described (3). Two-color immunofluorescence analysis of common marmoset PBL was performed using phycoerythrin-conjugated anti-NKH1 and FITC-conjugated anti-T11 or anti-T8 (Coulter Immunology, Hialeah, FL), as previously described (5). The cells were analyzed on an Epics V cell sorter (Coulter Electronics, Hialeah, FL).

Two-dimensional SDS-PAGE. Two-dimensional (nonreduced/reduced) SDS-PAGE of cell surface proteins was performed as previously described (7). In brief, radiolabeled total cell lysates (12 μl) were first resolved under nonreducing conditions in a 10% acrylamide gel. The gels were removed and equilibrated for 2 h at room temperature in reducing sample buffer (10% 2-ME, 100 mM DTT, 2% SDS, 0.08 M Tris-HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue). Samples were then electrophoresed under reducing conditions in a 10% acrylamide slab gel with 3% stacking gel. Gels were dried and visualized by autoradiography.

Results

NK Cell Activity of In Vitro H. Saimiri–immortalized Common Marmoset Lymphoid Cell Lines. Johnson and Jondal (8) had previously shown that cell lines derived from *H. saimiri*–induced lymphomas in New World primates exhibit NK activity. We therefore assessed the NK activity of a series of in vitro *H. saimiri*–immortalized cell lines to determine whether they too have NK function. These lines were studied for spontaneous cytotoxicity against various human NK-sensitive target populations (MOLT-4, MOLT-3 and K562) and a NK-resistant target population (Raji) using a specific ^{51}Cr-release assay (Fig. 1). All six in vitro *H. saimiri*–immortalized cell lines tested showed substantial cytotoxicity against MOLT-4 and MOLT-3, weak cytotoxicity against K562, and no cytotoxicity against Raji cells. These lines, therefore, exhibited NK function.

Phenotypic Analysis of H. Saimiri–immortalized Common Marmoset Lymphoid Cell Lines. Light microscopic examination of Wright-Giemsa–stained preparations of these lymphoid lines showed that significant heterogeneity existed in their morphologic appearance. However, the predominant cell type in these lines was LGL (data not shown).

As in our previous studies of such cell lines (3), the immortalized lymphocytes expressed the pan–T cell antigen T12 (Table I), but not the B cell–specific antigen B1 (data not shown). Every line expressed T8, and only 1 of 11 tested lines expressed detectable T4; that line, 39, contained only 15% T4$^+$ cells. Strikingly, cells immortalized by strain 11 *H. saimiri* expressed the NK cell–associated antigen NKH1. Another NK cell–associated antigen NKH2 was expressed by a minor population of cells in some lines. Therefore, these common marmoset cell lines express both T8 and NK–associated surface antigens. Inter-

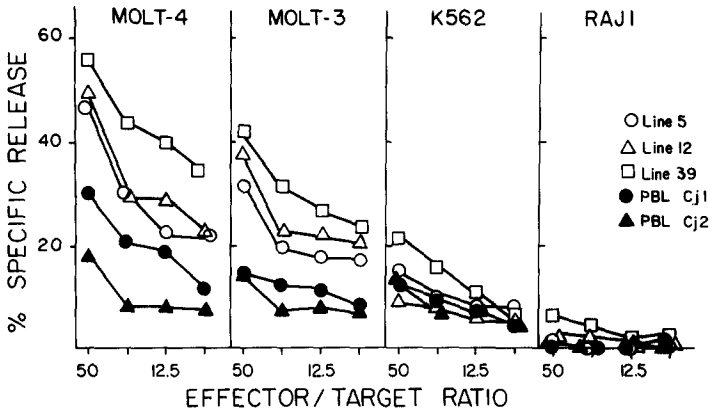

FIGURE 1. In vitro *H. saimiri*–immortalized common marmoset lymphoid lines exhibit NK cell activity. Percent specific release was determined in a 6-h killing assay using ^{51}Cr-labeled target cell populations. *Open symbols* represent cell lines and *closed symbols* represent common marmoset PBL.

TABLE I

Phenotypic Analysis of H. Saimiri-immortalized Common Marmoset Lymphoid Cell Lines

Line*	Percent of cells expressing each antigen‡					
	T12	T11	T4	T8	$NKH1_A$	NKH2
1	91 ± 5	14 ± 7	1 ± 1	71 ± 14	84 ± 7	8 ± 2
3	92 ± 3	20 ± 18	0 ± 0	72 ± 15	88 ± 2	6 ± 2
4	69 ± 28	55 ± 22	0 ± 0	60 ± 33	44 ± 23	18 ± 9
5	79 ± 13	5 ± 4	0 ± 0	66 ± 29	39 ± 14	10 ± 7
12	77 ± 18	13 ± 17	0 ± 0	72 ± 23	70 ± 19	7 ± 6
39	83 ± 18	58 ± 27	15 ± 13	84 ± 11	80 ± 4	9 ± 6

* All in vitro immortalizations were performed with *H. saimiri* strain 11.

‡ Data are expressed as percent cells staining positively, as determined by flow cytometry. Data are mean ± SD of three different measurements. Phenotyping of freshly isolated PBL from common marmoset blood shown: T12, 49 ± 10; T11, 57 ± 3; T4, 27 ± 5; T8, 21 ± 7; $NKH1_A$, 19 ± 13; NKH2, 5 ± 2; B1, 10 ± 9.

estingly, another pan–T cell structure, T11 (the sheep erythrocyte rosette receptor), was expressed to a variable extent by each cell line. The expression of NKH1 on these immortalized cell populations was confirmed by immunoprecipitation and gel electrophoresis (data not shown).

Expression of T Cell Receptor by H. Saimiri–immortalized Common Marmoset Cell Lines. Clones of human $NKH1^+$ NK cells have recently been shown to be of two distinct types: one expresses the T cell receptor and one does not (9). Available mAbs that recognize the human T cell receptor–associated T3 structure do not crossreact with that structure on common marmoset lymphocytes (4). We thus could not use cell-staining techniques to determine whether these cell lines expressed a T cell receptor. We therefore used two-dimensional SDS-PAGE to determine whether a T cell receptor–like structure was expressed on their cell surface. In a two-dimensional gel of an ^{125}I–surface labeled cell lysate in which the first dimension is run under nonreduced and the second under reduced conditions, T cell receptor proteins, which are disulfide-linked heterodimers, can be separated from the vast majority of membrane proteins as off-diagonal spots (7). As shown in Fig. 2, a surface-labeled lysate of the *H. saimiri*–

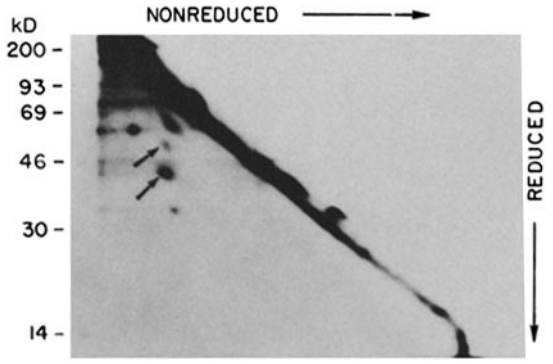

FIGURE 2. Two-dimensional SDS-PAGE (nonreduced and reduced) of a cell lysate from an *H. saimiri*–immortalized common marmoset line. Cells (line 39) were externally labeled with ^{125}I and the lysate was first resolved under nonreducing conditions in a 10% acrylamide gel. The gel was removed and equilibrated in a reducing buffer and subjected to electrophoresis in a second dimension under reducing conditions in a 10% acrylamide slab gel. The arrows indicate the position of the disulfide-linked heterodimer.

immortalized lymphoid line 39 showed off-diagonal spots which migrated with an apparent molecular weight of 90,000 under nonreducing conditions and 53,000 and 43,000 under reducing conditions. These are similar to the size of the human T cell receptor α and β chains. Additional experiments using three different *H. saimiri*–immortalized lines also showed similar off-diagonal spots (data not shown). Similar experiments using an EBV-transformed common marmoset B cell line failed to detect off-diagonal spots in this molecular weight range (data not shown). The results suggest that the *H. saimiri*–immortalized common marmoset $T8^+$ $NKH1^+$ cell lines express a complete T cell receptor on their surface.

Two-Color Immunofluorescence Analysis of Common Marmoset PBL. These data indicated that a highly restricted cell population predominated in the strain 11 *H. saimiri*–immortalized common marmoset cell lines: a functional NK cell which expresses T8, NKH1 and the T cell receptor. We then addressed the question of what proportion of PBL of the common marmoset this population represented. This was done using two-color immunofluorescence staining and flow cytometry analysis to determine the percent of common marmoset PBL expressing both T8 and NKH1. Common marmoset PBL were analyzed using mAbs conjugated directly with phycoerythrin or fluorescein. Only a minor proportion of $NKH1^+$ cells coexpressed the T8 antigen (Fig. 3*B*), whereas the majority of $NKH1^+$ cells coexpressed the T11 antigen (Fig. 3*A*). In fact, in repeated experiments, $\leq 3\%$ of common marmoset PBL were $NKH1^+$ $T8^+$. These results indicate that $T8^+$ $NKH1^+$ cells, the predominant cell in the *H. saimiri*–immortalized common marmoset lymphoid lines, are a very minor population of cells in the peripheral blood of common marmosets.

Discussion

The morphologically homogeneous peripheral blood LGL population of man is phenotypically quite heterogeneous. Human NK cells, while usually $T11^+$, consist of both $T8^+$ and $T8^-$ cells (10). Moreover, only a minor population of

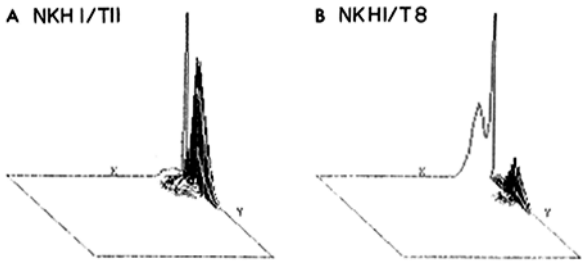

FIGURE 3. Two-color immunofluorescence analysis of common marmoset PBL. Cells were stained with both directly phycoerythrin-conjugated anti-NKH1 (abscissa) and a fluorescein-conjugated monoclonal antibody (either anti-T11 or anti-T8, ordinate) and analyzed on an Epics V. Percentages of cells expressing each antigen were: (*A*) $NKH1^+$ $T11^-$, 2%; $NKH1^+$ $T11^+$, 15%; $NKH1^-$ $T11^+$, 49%. (*B*) $NKH1^+$ $T8^-$, 11%; $NKH1^+$ $T8^+$, 3%; $NKH1^-$ $T8^+$, 17%.

human NK cells express T3, a T cell receptor–associated structure (5, 9). By analogy to these findings in humans, one would expect that the $NKH1^+$ $T8^+$ cell that expresses a T cell receptor would be only a subpopulation of the circulating NK cells in the common marmoset. We have in fact shown that it makes up a very small percentage of the common marmoset's PBL ($\leq$3%). It is therefore indeed striking that the predominant cell in *H. saimiri* strain 11 in vitro–immortalized common marmoset PBL is a rare lymphocyte that is $NKH1^+$ $T8^+$ and expresses the T cell receptor.

While these studies suggest that the immortalized lymphocyte populations are phenotypically restricted, heterogeneity clearly exists in the cell lines. The cells in individual lines differ in both their morphologic appearance and expression of T11. This heterogeneity may account for the finding that the immortalized lines show quantitatively less cytotoxicity than do IL-2-dependent NK clones.

Susceptible New World primates die after *H. saimiri* infection, with evidence of tumor masses, leukemia, or extensive parenchymal infiltration with lymphocytes. It is not clear to what extent these in vivo proliferating lymphocyte populations represent virally transformed cells or reactive lymphocytes. The present evidence that the virus-transformed lymphocytes are a phenotypically homogeneous population of a very restricted lymphocyte subpopulation will allow us to clarify this question through in situ phenotypic characterization of the tumor cells.

While other phenotypically distinct PBL populations may be potentially susceptible to immortalization by *H. saimiri* strain 11, the $NKH1^+$ $T8^+$ T cell receptor–positive lymphocyte of the common marmoset clearly predominates in these cell populations, and therefore appears to be most efficiently immortalized. The interactions that lead to the specificity of *H. saimiri* strain 11–induced lymphocyte immortalization should have important implications for the disease induced by this virus in New World monkeys.

Summary

Herpesvirus saimiri induces a fatal lymphoproliferative syndrome in a variety of New World primate species. We now show that cell lines derived from PBL of the common marmoset by in vitro–immortalization with *H. saimiri* strain 11 represent a remarkably restricted lymphocyte population. These cell lines have

NK cell function, phenotypically express both suppressor/cytotoxic (T8) and NK cell (NKH1)–associated antigens, and express a T cell receptor. This subpopulation of lymphocytes is a very minor population of cells in the peripheral blood of common marmosets (≤3%). The specificity in the interaction between *H. saimiri* strain 11 and a subpopulation of common marmoset lymphocytes represents an example of a restricted viral lymphotropism and may have important implications for the disease induced by this virus in New World monkeys.

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