Brief Definitive Report

A SUBSET OF MEMORY CD4+ HELPER T LYMPHOCYTES IDENTIFIED BY EXPRESSION OF Pgp-1

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The phenotypic distinction between naive T lymphocytes, before primary activation by antigen, and previously stimulated memory T cells has remained elusive until recently. Although several surface markers phenotypically subdivide $CD4^+$ or $CD8^+$ cells (1–6), or are transiently expressed by T cells upon activation (7–12), none of these determinants has been formally shown to identify a subset of murine memory T cells.

We recently observed that the cell surface determinant Pgp-1 (Ly-24) is heterogeneously expressed by murine peripheral T lymphocytes (13). Pgp-1 is a transmembrane 95-kD glycoprotein found on a wide variety of cell types besides lymphocytes, including bone marrow, lung, brain, and liver (14, 15). The molecule appears to be highly conserved among species (15), although its function remains unknown. Cell surface expression of Pgp-1 by T cells has been shown to be acquired at the time of primary antigenic stimulation and constitutively expressed thereafter (13). A prediction from this observation was that T cells responding to immunizing antigen in vivo would be found within the $Pgp\text{-}1^+$ subset. This was confirmed for the $CD8^+$ subset of murine T cells; after immunization with either of two antigens, specific cytolytic T lymphocytes were found nearly exclusively within the $Pgp\text{-}1^+$ subset (13, 16). The current study extends these observations to $CD4^+$ helper T lymphocytes (Th) by showing that the minor $Pgp\text{-}1^+$ subset of $CD4^+$ cells also contains the antigen-specific Th after immunization with either keyhole limpet hemocyanin (KLH) or sperm whale myoglobin (SWM).

Materials and Methods

mAbs. Rat mAbs directed against murine Pgp-1 (IM7.8.1) (15) and CD4 (GK 1.5) (17) were the gifts of R. Hyman (Salk Institute) and F. Fitch (University of Chicago), respectively.

Immunizations and Cell Preparations. Adult DBA/2 mice were immunized at the base of the tail with either KLH (75 μg in 100 μl CFA) or SWM (100 μg in 100 μl CFA). Inguinal lymph nodes (LN) were removed 8–10 wk later from four mice and single cell suspensions prepared by homogenization in RPMI medium containing 5% (vol/vol) FCS and 10 mM Hepes buffer. Cell viability was determined by trypan blue exclusion.

Flow Microfluorometry (FMF) and Cell Sorting. For two-color FMF, cells (10^7/ml) were stained (4°C for 30 min) sequentially with anti-Pgp-1 mAb, FITC-conjugated goat anti-rat Ig (GAR-FITC), followed by biotin-labeled anti-CD4 mAb and then Texas red–avidin. Samples were

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passed on a flow microfluorometer (FACS STAR; Becton Dickinson & Co., Mountain View, CA) gated to exclude nonviable cells by propridium iodide staining. At least 10^4 cells were accumulated for analysis. $CD4^+$ cells were sterilely sorted into $Pgp\text{-}1^+$ and $Pgp\text{-}1^-$ subsets.

Limiting Dilution Analysis. $CD4^+$ LN T cells, sorted into $Pgp\text{-}1^+$ and $Pgp\text{-}1^-$ subsets, were placed in limiting dilution culture at 24 replicates per dilution in round-bottomed microwells containing 7.5×10^5 irradiated (2,000 rad) DBA/2 spleen cells with appropriate antigen (KLH at 100 μg/ml or SWM at 20 μg/ml final concentration) or wells containing alloantigen $H\text{-}2^b$ (7.5×10^5 irradiated C57BL/6 spleen cells) in 200 μl culture medium (RPMI, 10% FCS supplemented with 50 U/ml human rIL-2) (Cetus Corp., Emeryville, CA). After a 10-d incubation (37°C, 5% CO_2), individual wells were visually scored for growth. Specificity was then determined by resuspending individual microcultures and dividing them equally in each of two new wells. Freshly irradiated DBA/2 spleen cells (7.5×10^5/well) were then added either in the absence or presence of antigens KLH or SWM without IL-2. Cultures were incubated 48 h longer and then pulsed with [^{3}H]thymidine during the last 12 h. Counts from each microculture without antigen were subtracted from the equivalent microculture containing antigen. Positive wells were defined as those exceeding the mean cpm in the absence of antigen by 3 SD. Minimal estimates of antigen-specific Th precursor frequency were derived by the χ^2 minimization method (18).

Results and Discussion

Subsets of Peripheral $CD4^+$ Cells Based upon Pgp-1 Expression. Two-color FMF demonstrated that LN $CD4^+$ T cells from normal, nonimmunized DBA/2 mice could be separated by Pgp-1 expression into a major (91% ± 5%) subpopulation of low-intensity staining cells ($Pgp\text{-}1^-$) and a minor (9% ± 5%) $Pgp\text{-}1^+$ subpopulation (mean ± SD of four experiments) (Fig. 1 *A*). In DBA/2 mice immunized 8–10 wk previously, LN $CD4^+$ cells showed an increased percentage of $Pgp\text{-}1^+$ lymphocytes. In KLH-immunized mice, the $Pgp\text{-}1^+$ subset represented 19% ± 1% of $CD4^+$ cells, twice normal, and in SWM-immunized mice, it represented 13%, a 39% increase relative to nonimmunized DBA/2 mice. This agrees with previous results for the $CD8^+$ subset in mice immunized with murine sarcoma virus or alloantigen, showing variously increased proportions of $Pgp\text{-}1^+$ cells (13). Presumably, this reflects the acquisition of Pgp-1 by an expanding population of antigen-specific T cells responding to the immunogen. However, as was found for $Pgp\text{-}1^+$ $CD8^+$ lymphocytes, the frequency of an antigen-specific T cell, even after immunization, is too low (see below) to account for all the increase in $Pgp\text{-}1^+$ $CD4^+$ cells after immunization. Hence, the enlarged $Pgp\text{-}1^+$ subset of T cells in immunized mice must partly rep-

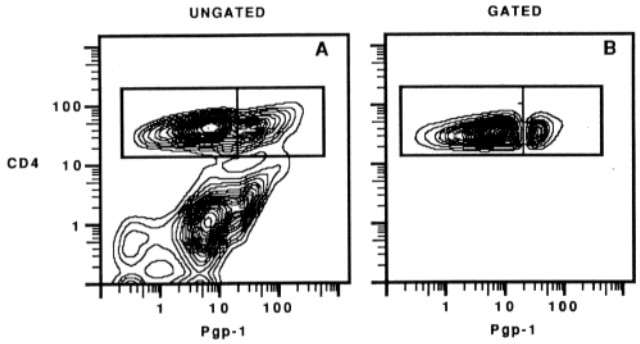

FIGURE 1. Pgp-1 expression by $CD4^+$ LN cells from DBA/2 mice (*A*). LN cells were stained sequentially with anti-Pgp-1 mAb, GAR-FITC, biotinylated anti-CD4 mAb, followed by avidin-Texas Red. $CD4^+$ cells were then gated and sorted into $Pgp\text{-}1^-$ and $Pgp\text{-}1^+$ subpopulations (*B*).

resent other factors (e.g., effect of adjuvant or secondary immune responses). Furthermore, the Pgp-1$^+$ subset contains a minor (10–20%) population of large blast-like T lymphocytes not observed in the Pgp-1$^-$ subset (13). Whether these large Pgp-1$^+$ T cells represent recently activated cells remains to be determined.

Frequencies of Antigen-specific Th Cells in the Pgp-1 Subsets. To examine whether the functional significance of heterogeneous expression of Pgp-1 by CD4$^+$ cells paralleled that observed for CD8$^+$ cells, we examined the frequencies of antigen-specific responses in cell-sorted Pgp-1$^+$ and Pgp-1$^-$ subsets of CD4$^+$ cells from mice immunized with KLH or SWM (Fig. 1 *B*). Fig. 2 *A* illustrates the limiting dilution analysis results from mice immunized 8–10 wk previously with KLH. As shown, the antigen-specific Th precursor frequency for the Pgp-1$^+$ subset of CD4$^+$ cells was 1:1,100, compared with 1:17,400 for the Pgp-1$^-$ subset, representing a nearly 16-fold difference. In a similar manner, from SWM-immunized mice, there was an enrichment of at least 11-fold in the frequency of antigen-specific Th between the Pgp-1$^+$ (1:3,570) and Pgp-1$^-$ (<1:40,000) subsets (Fig. 2 *C*). These findings were consistent on repeat experiments (Table I).

The enhanced frequency of response in the Pgp-1$^+$ subset was not the result of an inability of Pgp-1$^-$ cells to respond to nonimmunizing antigen, as evidenced by the similar frequencies of response in both Pgp-1$^+$ and Pgp-1$^-$ subsets to alloantigen (Fig. 2, *B* and *D*). This suggests that the enhanced frequency of antigen-specific response in the Pgp-1$^+$ subset was confined to the immunizing antigen. Furthermore,

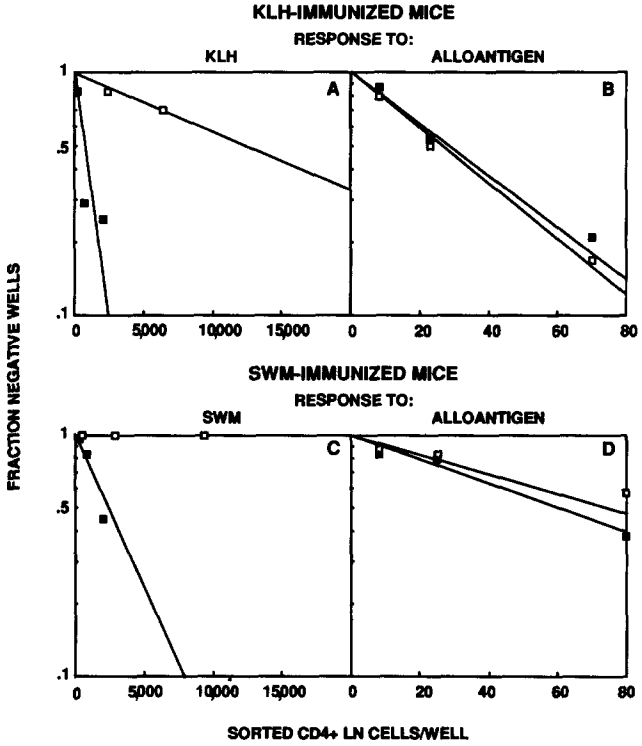

FIGURE 2. Comparison of frequencies of antigen-specific (*A* and *C*) vs. allospecific (*B* and *D*) Th precursors in the Pgp-1$^-$ (□) and Pgp-1$^+$ (■) subpopulations of CD4$^+$ cells from either KLH-immunized (*A* and *B*) or SWM-immunized (*C* and *D*) mice. Frequencies are summarized in Table I.

TABLE I

Frequencies of Antigen-specific and Allospecific Th Precursors in Pgp-1 Subsets of CD4⁺ LN Cells

Immunizing antigen	Exp.	Antigen-specific Th frequency^{-1}			Allospecific (H-2^b) Th frequency^{-1}	
		Pgp-1$^-$	Pgp-1$^+$	Pgp-1$^-$ + Pgp-1$^+$*	Pgp-1$^-$	Pgp-1$^+$
KLH	1	17,400	1,100		42	45
	2	<40,000	1,175			
	3	32,000	640	585		
SWM	1	<40,000	3,750		116	90
	2	<40,000	5,300			

* CD4⁺ cells were sorted into Pgp-1$^-$ and Pgp-1$^+$ subsets and then remixed at a 2:1 ratio of Pgp-1$^-$ to Pgp-1$^+$ cells before being placed in limiting dilution microcultures. The Th frequency is calculated based upon the number of Pgp-1$^+$ cells plated.

the enhanced frequency of antigen-specific response in the Pgp-1$^+$ subset was not a result of the absence of suppression by the Pgp-1$^-$ subset. When sorted Pgp-1$^-$ and Pgp-1$^+$ cells were remixed at a 2:1 ratio of Pgp-1$^-$ to Pgp-1$^+$ cells, the response frequency was equal to that of Pgp-1$^+$ cells alone (Fig. 3, Table I).

These current studies extend earlier work on the phenotypic identification of memory cytolytic T lymphocytes by showing that Pgp-1 is also a surface marker of memory CD4⁺ Th cells. The accumulated data to date suggest that naive mature thymocytes emerge from the thymus lacking expression of Pgp-1. The absence of cell surface Pgp-1 on nearly all mature medullary-type (CD4⁺8$^-$, CD4$^-$8⁺) thymocytes is consistent with this model (13). Also in support of this notion is that after thymectomy, Pgp-1$^-$ cells gradually disappear in peripheral lymphoid tissues with a resulting accumulation of Pgp-1$^+$ cells (13). Furthermore, all T cells from athymic nude mice express Pgp-1$^+$ (13). After primary antigenic stimulation in vitro, Pgp-1$^-$ cells are induced to express Pgp-1 constitutively thereafter. As a result, after immunization in vivo, antigen-specific CD8⁺ or CD4⁺ T cells are enormously enriched within the minor Pgp-1$^+$ subset.

That induction of Pgp-1 expression by T cells should be linked to antigenic stimulation via the TCR is of considerable interest. We recently observed that when naive T cells acquire surface Pgp-1, they also become high producers of IFN-γ and IL-3

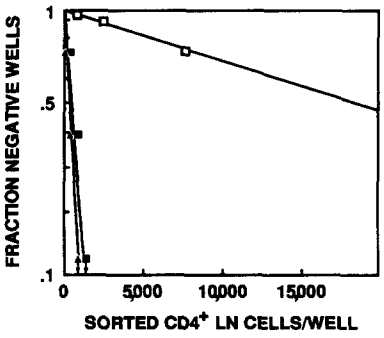

FIGURE 3. Comparison of KLH-specific Th frequencies in Pgp-1$^-$ (□), Pgp-1$^+$ (■), or a mixture of Pgp-1$^-$ and Pgp-1$^+$ (2:1) (▲) CD4⁺ LN cells from KLH-immunized mice. Frequency analysis for the Pgp-1$^-$ + Pgp-1$^+$ mixture is based upon the numbers of plated Pgp-1$^+$ cells. Frequencies are summarized in Table I.

(19). Thus, the gene regulation of certain surface molecules, as well as particular lymphokines, appears to be closely linked to primary T cell activation via the TCR. These data demonstrate that several events in T cell development may occur extrathymically.

The function of the Pgp-1 molecule is currently unknown although some evidence suggests it may be involved in motility of fibroblasts (20). We have not observed any effect of anti-Pgp-1 antibody on either proliferation or cytolytic effector function (R. C. Budd, unpublished observations). However, recently, mAbs against either Pgp-1 or LFA-1 were shown to inhibit platelet-dependent cytotoxicity of antibody-coated sheep erythrocytes (21). Thus, at least in certain situations, Pgp-1 may play a role in cell-cell adhesion.

Summary

The Pgp-1 glycoprotein (Ly-24 antigen) is acquired by mature murine T lymphocytes at the time of primary antigen stimulation. Pgp-1 was previously shown to be a useful cell surface marker for distinguishing antigen-specific memory $CD8^+$ T lymphocytes after immunization. Here we demonstrate that this observation extends to $CD4^+$ T lymphocytes. Antigen-specific $CD4^+$ T cells in mice immunized with sperm whale myoglobin or keyhole limpet hemocyanin were contained nearly exclusively in the minor Pgp-1^+ subset.

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References

1. Gatenby, P. A., G. S. Kansas, C. Y. Xian, R. L. Evans, and E. G. Engleman. 1982. Dissection of immunoregulatory subpopulations of T lymphocytes within the helper and suppressor sublineages in man. *J. Immunol.* 129:1997.
2. Damle, N. K., N. Mohagheghpour, G. S. Kansas, D. M. Fiswild, and E. G. Engleman. 1985. Immunoregulatory T cell circuits in man. Identification of a distinct T cell subpopulation of the helper/inducer lineage that amplifies the development of alloantigen-specific suppressor T cells. *J. Immunol.* 134:235.
3. Clavo, C. H., A. Bernard, S. Huet, E. Le Roy, L. Boumsell, and A. Senik. 1986. Regulation of immunoglobulin synthesis by human T cell subsets as defined by anti-D44 monoclonal antibody within the $CD4^+$ and $CD8^+$ subpopulations. *J. Immunol.* 136:1144.
4. Morimoto, C., N. L. Letvin, J. A. Distaso, W. R. Aldrich, and S. F. Schlossman. 1985. The isolation and characterization of the human suppressor inducer T cell subset. *J. Immunol.*. 134:1508.
5. Reinherz, E. L., C. Morimoto, K. A. Fitzgerald, R. E. Hussey, J. F. Daley, and S. F. Schlossman. 1982. Heterogeneity of human $T4^+$ inducer T cells defined by a monoclonal antibody that delineates two functional subpopulations. *J. Immunol.* 128:463.
6. Arthur, R. P., and D. Mason. 1986. T cells that help B cell responses to soluble antigen are distinguishable from those producing interleukin 2 on mitogenic or allogeneic stimulations. *J. Exp. Med.* 163:774.
7. Cantrell, D. A., and K. A. Smith. 1983. Transient expression of interleukin 2 receptors. Consequences for T cell growth. *J. Exp. Med.* 158:1895.

8. Trowbridge, I. S., and M. B. Omary. 1981. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc. Natl. Acad. Sci. USA.* 78:3039.
9. Helderman, J. H., and T. B. Strom. 1979. Role of protein and RNA synthesis in the development of insulin binding sites on activated thymus-derived lymphocytes. *J. Biol. Chem.* 254:7203.
10. Evans, R. L., T. J. Faldetta, R. E. Humphreys, D. M. Pratt, E. J. Yunis, and S. F. Schlossman. 1978. Peripheral human T cells sensitized in mixed leukocyte culture synthesize and express Ia-like antigens. *J. Exp. Med.* 148:1440.
11. Haynes, B. F., M. E. Hemler, D. L. Mann, G. S. Eisenbarth, J. Shelhamer, H. S. Mostowski, C. A. Thomas, J. L. Strominger, and A. S. Fauci. 1981. Characterization of a monoclonal antibody (4F2) that binds to human monocytes and to a subset of activated lymphocytes. *J. Immunol.* 126:1409.
12. Cotner, T., J. M. Williams, L. Christenson, H. M. Shapiro, T. B. Strom, and J. Strominger. 1983. Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. *J. Exp. Med.* 157:461.
13. Budd, R. C., J. -C. Cerottini, C. Horvath, C. Bron, T. Pedrazzini, R. C. Howe, and H. R. MacDonald. 1987. Distinction of virgin and memory T cells. Stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J. Immunol.* 138:3120.
14. Hughes, E. N., G. Mengod, and J. T. August. 1981. Murine cell surface glycoproteins. Characterization of a major component of 80,000 daltons as a polymorphic differentiation antigen of mesenchymal cells. *J. Biol. Chem.* 256:7023.
15. Trowbridge, I. S., J. Lesley, R. Shulte, R. Hyman, and J. Trotter. 1982. Biochemical characterization and cellular distribution of a polymorphic, murine cell-surface glycoprotein expressed on lymphoid cells. *Immunogenetics.* 15:229.
16. Budd, R. C., J. -C. Cerottini, and H. R. MacDonald. 1987. Phenotypic identification of memory cytolytic T lymphocytes in a subset of Lyt-2^+ cells. *J. Immunol.* 138:1009.
17. Dialynas, D. P., Z. S. Quan, K. A. Wall, A. Piernes, J. Quitand, M. R. Loken, M. Piernes, and F. W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human leu-3/T4 molecule. *J. Immunol.* 131:2445.
18. Ryser, J. E., and H. R. MacDonald. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. I. Comparison of precursor frequencies for proliferative and cytolytic responses. *J. Immunol.* 122:1691.
19. Budd, R. C., J. -C. Cerottini, and H. R. MacDonald. 1987. Selectively increased production of interferon-gamma by subsets of Lyt-2^+ and L3T4^+ T cells identified by expression of Pgp-1. *J. Immunol.* 138:3583.
20. Jacobson, K., D. O'Dell, B. Holifield, T. L. Murphy, and J. T. August. 1984. Redistribution of a major cell surface glycoprotein during cell movement. *J. Cell. Biol.* 99:1613.
21. McCaffery, P. J., A. S. Tan, and M. V. Berridge. 1987. Polymorphic glycoprotein-1 on mouse platelets: possible role of Pgp-1 and LFA-1 in antibody-dependent platelet cytotoxicity involving complement. *Blood.* 69:211.