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-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-1+ subset (13, 16). The current study extends these observations to CD4+ helper T lymphocytes (Th) by showing that the minor Pgp-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

\textbf{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.

\textbf{Immunizations and Cell Preparations.} Adult DBA/2 mice were immunized at the base of the tail with either KLH (75 \mu g in 100 \mu l CFA) or SWM (100 \mu g in 100 \mu l 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.

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

Limiting Dilution Analysis. CD4$^+$ LN T cells, sorted into Pgp-1$^+$ and Pgp-1$^-$ subsets, were placed in limiting dilution culture at 24 replicates per dilution in round-bottomed microwells containing $7.5 \times 10^5$ irradiated (2,000 rad) DBA/2 spleen cells with appropriate antigen (KLH at 100 $\mu$g/ml or SWM at 20 $\mu$g/ml final concentration) or wells containing alloantigen H-2$^b$ ($7.5 \times 10^5$ irradiated C57BL/6 spleen cells) in 200 $\mu$l 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 ($7.5 \times 10^5$/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 $\chi^2$ 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-1$^-$) and a minor (9% ± 5%) Pgp-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-1$^+$ lymphocytes. In KLH-immunized mice, the Pgp-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-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-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-1$^+$ CD4$^+$ cells after immunization. Hence, the enlarged Pgp-1$^+$ subset of T cells in immunized mice must partly rep-
represent 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,
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.
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.

We thank Tim Knack for operation of the cell sorter, Mark Koch, and Leona Daidone for preparation of the manuscript.

Received for publication 23 September 1988 and in revised form 15 December 1988.

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