Reciprocal Expression of P-Glycoprotein and TAP1 Accompanied by Higher Expression of MHC Class I Antigens in T Cells of Old Mice

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We have shown previously that (a) aging leads to an increase in the proportion of murine splenic T cells that express high activity of P-glycoprotein (PGP), the ATP-dependent plasma membrane pump that mediates multiple drug resistance, and (b) PGP

\textsuperscript{+} CD4 memory cells from mice of any age do not proliferate or secrete IL-4 after activation with anti-CD3 and IL-2. We now report that the age-associated increase in expression of MHC Class I molecules is limited to the subset of T cells that overexpress PGP and thus extrude the fluorochrome R123 (the "R123

\textsuperscript{+} " subset). Although H-2 levels increase on T cells of old mice, the levels of TAP1, a component of the polypeptide pump responsible for assembly and internal transport of Class I MHC molecules, decline, unexpectedly, by about fourfold in T cells from old donors. Thus, aging leads to reciprocal changes in the level of T-cell expression of PGP and TAP1, two closely related members of the ABC superfamily of peptide transport proteins.

Aging in mice and humans is accompanied by shifts in the proportions of phenotypically distinguishable T-cell subsets. The most prominent of these is the increase in the proportion of T cells expressing the antigenic profile characteristic of memory cells, as determined by expression of CD44, CD45RB, and MEL-14, and the corresponding decrease in the proportion of T cells with the phenotype of naive cells (De Paoli et al., 1988; Lerner et al., 1989; Ernst et al., 1990; Nagelkerken et al., 1991; Pilarski et al., 1991). Some of the functional differences between T cells from young and old mice can be accounted for by the shift from naive to memory cells, including declining responses to mitogenic plant lectins (Lerner et al., 1989), diminished production of IL-2 and IL-3 (Flurkey et al., 1992), and increased resistance to calcium signal generation (Philosophe and Miller, 1990). In other cases, however, functional changes with age cannot be ascribed to the accumulation of memory T cells and loss of naive cells, but instead reflect age-dependent alterations within one or both of these subsets. After activation by anti-CD3 antibody, for example, memory (but not naive) T cells are capable of vigorous proliferation and IL-4 production in the presence of IL-2, but memory T cells isolated from old donors are far less active than memory T cells from young mice in this assay (Li and Miller, 1993).

In search of ways to reveal unsuspected heterogeneity within the naive and memory cell subsets that might contribute to age-dependent functional changes, we recently noted an increase with age in the proportions of T cells that could extrude the fluorochrome Rhodamine-123 (R123) (Witkowski and Miller, 1993). Extrusion of R123 was shown (Witkowski and Miller, 1993) to be inhibitable by each of four unrelated inhibitors of P-glycoprotein (PGP), the 170 kD, ATP-dependent plasma membrane pump that mediates multiple-drug resistance (Juranka et al., 1989; Gros and Buschman, 1993). The proportion of cells that could extrude R123 ("R123

\textsuperscript{+} " cells"), presumed to express high levels of PGP, was found to increase more than twofold with aging. R123

\textsuperscript{+} and R123

\textsuperscript{0} T cells were present within both the memory (CD44

\textsuperscript{+}) and naive (CD44

\textsuperscript{0}) pools of both CD4 and CD8 subsets, and the R123

\textsuperscript{+} cells showed an age-associated increase in all four T-cell subpopulations. When R123

\textsuperscript{+} and R123

\textsuperscript{0} T cells were isolated from the CD4 memory pool, only the R123

\textsuperscript{+} cells were able to proliferate and secrete IL-4 after activation with anti-CD3 and expansion in IL-2. The accumulation of R123

\textsuperscript{+} T cells and relative decline of R123

\textsuperscript{0} cells within the CD4 memory pool accounts for some, although not all, of the age-related decline in IL-2 dependent proliferation and IL-4 production in CD3-activated CD4 memory T cells (Witkowski et al., 1994). Thus PGP expression and R123 extrusion reflect an age-dependent, functionally significant subdivision of the naive and memory subsets.

PGP is a member of the ABC superfamily of ATP-dependent transporter proteins, and within this superfamily is most closely related to TAP1 and TAP2, which as a heterodimer participate in the loading of peptides into the presentation cleft of the Class I MHC molecule, and the translocation of the peptide/MHC complex from the cytoplasmic to the luminal face of the endoplasmic reticulum (Monaco et al., 1990; Neefjes et al., 1993; Ortmann et al., 1994). Furthermore, studies of mutant cells that lack TAP1 and/or TAP2 show defects in antigen presentation and MHC protein expression that can be restored by transfection with the missing transporter gene (Powis et al., 1991; Van Kaer et al., 1992). Thus, the TAP1/TAP2 heterodimer plays a key role in the processing and presentation of peptides.
role in antigen presentation and assembly of Class I MHC molecules.

Two groups have previously reported an increase in the expression of Class I MHC molecules in T cells of old mice (Sidman et al., 1987; Janick-Buckner et al., 1991). Since PGP is structurally similar to TAP1 and TAP2 (50%-70% sequence similarity at the nucleic acid level), and like TAP1/TAP2 has the ability to transport biologically active peptides through membranes (Raymond et al., 1992; Sharma et al., 1992), we considered the possibility that the age-dependent increase in T-cell Class I MHC molecules might reflect increased activity of the TAP1/TAP2 transporter or a possible role for PGP itself in MHC assembly. We report here that the age-dependent increase in Class I H-2 expression is limited to the R12310 T-cell subset. Furthermore, we report that aging leads, unexpectedly, to a decline in the amounts of TAP1 present within T cells along with the reciprocal increase in PGP levels.

**Materials and Methods**

*Animals.* — Male CB6F1 mice were obtained from the NIA contract colony at the Charles River Breeding Laboratories (Kingston, NY). Animals between 3-4 months were classified as young, between 12-14 months as middle-aged, and between 18-22 months of age as old. The mice were housed in specific-pathogen free conditions, and not used if they exhibited signs of illness, including splenomegaly, skin lesions, or grossly visible tumors.

*Cell preparations.* — Mice were sacrificed by CO₂ asphyxiation, and their spleen cells were released by gentle pressure of tissue fragments between frosted glass microscope slides in Hank’s Balanced Salt Solution containing 0.2% bovine serum albumin (HBSS-BSA). Mononuclear cells were obtained by flotation over Lympholyte-M (Cedarlane). The suspension was then enriched in T cells by panning on anti-mouse Ig coated plate, and depleted of macrophages by incubation in plastic tissue culture flasks for 1 hour at 37 °C.

*Staining and flow cytometry.* — Cells were stained differentially with R123 as previously described (Witkowski and Miller, 1993). Briefly, cells were suspended at 4 x 10⁶/ml and incubated with 6.5 µM R123 for 10 minutes at 37 °C, washed, and incubated in dye-free medium for another 30 minutes at 37 °C. This procedure allowed PGP⁺ cells to expel most of the fluorochrome.

For simultaneous analysis of R123 and H-2, cells were stained with R123 as previously described (Witkowski and Miller, 1993). Briefly, cells were suspended at 4 x 10⁶/ml and incubated with 6.5 µM R123 for 10 minutes at 37 °C, washed, and incubated in dye-free medium for another 30 minutes at 37 °C. This procedure allowed PGP⁺ cells to expel most of the fluorochrome.

Detection of TAP1 expression by Western blot. — T cell-enriched, macrophase-depleted preparations were washed three times in protein-free HBSS, and the cell pellet lysed in 3% Nonidet P40 lysis buffer at 2 x 10⁶ cells/ml for 1 hour on ice. Insoluble debris was removed by centrifugation and the supernatants stored at -70 °C. Prior to the analysis, thawed samples were mixed 1:1 with reducing sample buffer and boiled for 7 minutes. Proteins were then resolved by SDS-10% polyacrylamide gel electrophoresis under reducing conditions. Lysates corresponding to 2 x 10⁶ cells were loaded per lane. Lysates of the cell lines RMA and RMA-S (a mutant with a stop codon in the TAP2 gene) were obtained from Dr. Young Yang, Scripps Research Institute, La Jolla, CA, and were used as controls. Resolved proteins were electrochemically transferred to nitrocellulose membrane (Schleicher-Schuell). Free protein-binding sites were blocked with 5% bovine albumin and the membranes incubated with 1:1500 rabbit anti-mouse TAP1 antisera (the gift of Dr. Yang). After extensive washing with PBS containing 0.2% Tween 20, and incubation with secondary antibody (peroxidase-conjugated anti-rabbit Ig, Amersham) the membranes were processed for detection of bound antibodies by enhanced chemiluminescence (ECL kit, Amersham).

**Immunoprecipitation analysis of TAP1.** — Spleen cells were depleted of erythrocytes on Lympholyte M, depleted of
B cells by panning, and then lysed in 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 20 mM Tris, pH 7.2. Lysates were incubated on ice for 30 minutes and then centrifuged at 15,000 rpm (microfuge) for 15 minutes to remove insoluble debris. For analysis of TAP1 levels, lysates were incubated with a 1:1000 dilution of rabbit anti-mouse TAP1 serum overnight at 4 °C. The beads were washed with lysis buffer (3 X) and with 10 mM Tris (pH 7.2), suspended in a 1:1 mixture of lysis and sample buffer, and boiled for 7 min prior to electrophoretic resolution of proteins on an 8% polyacrylamide gel. Separated proteins were electrophoretically transferred to nitrocellulose, and the membranes blocked with 5% BSA/PBS for 2 hr at room temperature. The blots were then incubated with rabbit anti-TAP1 antiserum (1:1500) in 1% BSA/PBS overnight at 4 °C. After extensive washing with PBS/0.5% Tween 20, the membrane was incubated with a 1:2000 dilution of peroxidase labeled goat anti-rabbit Ig (Amersham) for 2 hr at room temperature, washed extensively, and developed using enhanced chemiluminescence (Amersham). TAP1 expression was then quantified by scanning densitometry of the exposed films.

Western blot analysis of PGP levels. — NP-40 lysates were prepared from spleen cells and from cells of the PGP-expressing, vinblastine-resistant line CEM/VBL and its parental line. PGP levels were analyzed using the method described above for TAP1 immunoprecipitation, except for the use of 1 μg purified rabbit anti-mdr antibody (Oncogene Science) as the precipitating reagent, 1 μg of monoclonal antibody C219 (Signet Laboratories) as the detection reagent, and the substitution of peroxidase labeled sheep anti-rabbit Ig for anti-rabbit Ig as the developing reagent.

RESULTS

Expression of Class I MHC (H-2) antigens and β2-microglobulin increases in splenic T cells of aged mice. — The expression of MHC Class I antigens was evaluated by flow cytometry in spleen T cells of young (3 months) and old (> 20 months) CB6F1 mice. We used in this series of tests two antibodies specific for D, two specific for D, and one specific for K. Class I MHC expression was found to be increased by aging whether the comparison involved peak (modal) channel, mean fluorescence intensity, or median channel number. Figure 1 shows mean fluorescence intensity levels (± SEM) for each of the five anti-Class I antibodies examined; the age effects are statistically significant by t-test for all four of the anti-D reagents. The within-experiment ratios (Young:Old) of mean fluorescence intensity for these five antibodies varied between 1.34 and 1.62, and were statistically significant (p < .04) for all five, including the anti-K reagent (data not shown). Splenic T cells from old mice also expressed higher levels of cell surface β2-microglobulin (antibody S19.8 in Figure 1, p < .05). Within experiments, the Young:Old ratio for β2-microglobulin levels was 1.24 ± 0.02 (p < .01). Thus, the effects of age on Class I MHC protein expression are small, but consistent and statistically significant. These results are in good agreement with data published previously by other laboratory groups (Sidman et al., 1987; Janick-Buckner et al., 1991). Preliminary experiments (not shown) revealed no difference in H-2 expression between CD4 and CD8 cells of young mice. Old CD8 cells expressed higher levels of H-2 than did CD4 cells from old mice. Aging led to an increase in H-2 expression within both the CD4 and CD8 subclasses.

H-2 expression is elevated only within the R123hi subsets of old mice. — Since P-glycoprotein is closely related to the TAP1/TAP2 heterodimer required for MHC Class I protein assembly, and since we had recently noted an age-dependent increase in T-cell extrusion of the P-glycoprotein substrate R123, we determined whether H-2 expression might differ between R123hi and R123' T cells of old or young mice. Splenic T cells were stained with R123, incubated to permit R123 extrusion, and then stained with antibodies to H-2 before examination by flow cytometry. The results (Figure 2) showed that while both R123hi and R123' T cells of old or young mice. Splenic T cells were stained with R123, incubated to permit R123 extrusion, and then stained with antibodies to H-2 before examination by flow cytometry. The results (Figure 2) showed that while both R123hi and R123' T cells of old mice expressed equal (low) levels of all three H-2 antigens examined (D, D, and K), the R123' and R123' T cells of old mice differed in H-2 expression such that nearly all of the cells expressing high levels of H-2 antigens were within the R123' T cell subset. The proportion of H-2' T cells within the R123' subsets was low, and did not change with age, while the R123' subset, with higher P-glycoprotein activity (Witkowski and Miller, 1993), contained H-2' cells whose numbers increased with age.
We carried out a statistical analysis of a series of such experiments involving analysis of D\textsuperscript{d} and D\textsuperscript{b} expression; in each analysis we used three pairs of young and old mice. The proportion of T cells that were both H-2\textsuperscript{hi} and R123\textsuperscript{10} increased with age from 2%-3% in young mice to 15%-24% in old mice, while the proportion of T cells that were both H-2\textsuperscript{w} and R123\textsuperscript{w} showed no age-related change, remaining between 3% and 6%. There is a statistically significant increase with age in the H-2\textsuperscript{w}, R123\textsuperscript{10} subset, but not in the H-2\textsuperscript{hi}, R123\textsuperscript{hi} subset. Correspondingly, the proportion of the R123\textsuperscript{hi} cells that express the H-2\textsuperscript{w} phenotype also increases with age, e.g., from 12.5% ± 3.0 for H-2D\textsuperscript{d} in young mice to 35.9% ± 4.2 for old mice (p < .04). Thus, the H-2\textsuperscript{w} cells are largely restricted to the R123\textsuperscript{10} subsets of both young and old mice, and the increase with age in H-2\textsuperscript{w} cells is due both to an increase in the proportion of R123\textsuperscript{10} cells and an increase in the proportion of R123\textsuperscript{w} cells with higher levels of H-2 expression.

In a separate set of experiments (not shown), we used electronic cell sorting to prepare enriched populations of R123\textsuperscript{w} and R123\textsuperscript{10} T cells from young and old mice, and then stained these subpopulations with antibodies to D\textsuperscript{d}. We observed that old R123\textsuperscript{w} cells expressed higher levels of H-2 compared to R123\textsuperscript{10} cells from old mice, but that there was no difference in H-2 expression between R123\textsuperscript{w} and R123\textsuperscript{10} cells of young mice. These experiments supported our observations from the analytical flow cytometry (Figure 2). We conclude that aging leads to an increase in H-2 expression seen only within the R123\textsuperscript{w} T-cell population.

**Decreased levels of TAP1 in T cells from old mice.** — We considered the possibility that increased levels of Class I H-2 proteins at the surface of T cells from old mice might reflect an age-associated increase in cellular content of TAP1, which with TAP2 promotes the intracellular assembly of H-2 with \beta-2-microglobulin and internally generated peptides. Figure 3 shows representative Western blots using an antiserum specific for TAP1. Blotting with anti-TAP1 revealed a prominent single band at 74 KD in samples from young mice (lanes A-C). This band was also prominent in blots of both RMA and RMA-S cells (lanes G and H). Equivalent samples from old mice (lanes D-F) showed much lower expression of TAP1. Figure 4 summarizes the quantitative data from a series of such experiments using young, middle-aged, and old mice. T-cell TAP1 levels were found to be significantly lower in old mice compared to young mice.

To confirm the impression from the Western blotting experiments, we also carried out a series of immunoprecipitations using anti-TAP1 antibody with and without inhibition by specific peptide. The data are summarized in Figure 5. There is a statistically significant difference (p < .02; n = 3 pairs) between young and old mice in immunoprecipitable TAP1. TAP1 immunoprecipitation was, as expected, diminished (two- to threefold in young mice) by inclusion of a complementary peptide. We conclude that an increase in TAP1 expression is not likely to explain the age-related increase in H-2 Class I expression. Moreover, the data suggest unexpectedly that, in fact, expression of TAP1 declines in T cells from old mice.

**Increased levels of P-glycoprotein in T cells from old mice.** — To determine whether the increased functional activity of P-glycoprotein in T cells of old mice (Witkowski and Miller, 1993) reflected an increase in the amount of P-glycoprotein in these cells, lysates of T cells of young, middle-aged, and old mice were resolved by SDS-PAGE and blotted with an anti-P-glycoprotein antibody. As controls we used the vinblastine-resistant cell line CEM/VBL,
which expresses high levels of P-glycoprotein, and the parental line CEM. A representative experiment is shown in Figure 6. Antibody to P-glycoprotein reveals a diffuse band at approximately 170 kD in CEM/VBL cells, which is not detectable in CEM. A similar band is detectable in lysates of freshly isolated T cells, with higher amounts present in T cells from older donors. Figure 7 summarizes a series of 8 such experiments, showing an age-dependent increase in P-glycoprotein levels. On average, T cells from old mice contained threefold more P-glycoprotein than T cells from young donors; the difference between old and young mice is statistically significant by the Student-Newman-Keuls test at \( p < .05 \).

**DISCUSSION**

Our principal findings are that T cells from old mice have about threefold higher amounts of PGP than T cells from young mice; that increased H-2 Class I expression is restricted to the R123\( ^{hi} \) (i.e., PGP\( ^{hi} \)) T-cell subsets that accumulate in old mice; and that TAP1 levels decline about fourfold in T cells from old donors.
It seems likely that the increased amount of PGP in old T cells accounts for some (or perhaps all) of their increased ability to extrude R123. Our earlier report (Witkowski and Miller, 1993) included a limited amount of immunofluorescent data suggesting that T cells from young and old mice might have equivalent amounts of PGP protein, and thus that increased R123 extrusion by PGP might reflect postranslational modifications. In retrospect, these immunofluorescent data now seem likely to reflect nonspecific binding. Several lines of evidence suggest strongly that PGP is the principal agent for extrusion of R123. Extrusion of R123 by freshly isolated human and mouse T cells can be blocked by a diverse set of pharmacologically distinct PGP inhibitors (Coon et al., 1991; Chaudhary et al., 1991; Witkowski and Miller, 1993), and by antibodies to PGP (Chaudhary et al., 1992; Gupta et al., 1992a). Furthermore, human CD4 cell lines that do not express mdr1 mRNA are also unable to extrude R123 (Schluesener et al., 1992). T-cell activation by PHA leads to increases both in PGP and in the corresponding mdr1 mRNA (Gupta et al., 1992a). There is, however, also strong evidence that increased PGP function can result from PGP phosphorylation, particularly by protein kinase C (Bates et al., 1992; Fan et al., 1992; Gupta et al., 1992b; Ahmad and Glazer, 1993; Blobe et al., 1993), and that aging leads to changes in patterns of PK-C mediated phosphorylation in T cells (Proust et al., 1987; Patel and Miller, 1992). Thus our Western blot and immunoprecipitation data are consistent with the idea that the R123'° T cells that accumulate in old age have higher levels of PGP, but cannot yet rule out the idea that altered PGP phosphorylation may also contribute to the R123'° phenotype.

Our measurements of H-2 levels confirm previous work of others (Sidman et al., 1987; Janick-Buckner et al., 1991), but extend earlier results by showing that only the R123'° T cells of old mice show the increase in cell surface expression of MHC Class I proteins and β2-microglobulin. The amount of the increase is small, but consistent and statistically significant; its functional significance in relation to T-cell interactions with other cells is unknown. Class I molecules are expressed to varying degrees by nearly all somatic cells, and play an important role in presenting viral antigens, tumor antigens, and internally synthesized protein determinants to CD8+ T cells. Although it is possible that recognition of Class I-presented peptides on T-cell surfaces might play a role in immunoregulation, it seems unlikely that the small changes in expression that we have documented would materially alter T-cell immunity. More work will be needed to determine whether the increased levels of H-2 represent alterations in mRNA production, protein translation or assembly, or rates of shedding or degradation. Work in another laboratory (Janick-Buckner et al., 1991) has suggested that aging may lead to a 10-fold increase in H-2 mRNA levels, which may indicate alterations at several levels in the production of the mature H-2 surface protein. Although it is not known whether the levels of TAP1 and TAP2 present in the T cells of old mice are sufficient to support normal levels of H-2 assembly and peptide loading, it seems possible that some portion of the H-2 molecules in old T cells may be empty or misassembled, with lower half-life once inserted into the membrane.

We do not yet have a satisfying model that would account for the concomitant increase in Class I MHC expression, increase in PGP, and decrease in TAP1 we observe in T cells from old mice, and it is certainly possible that these phenomena may be coincident rather than causally linked. If one speculates that PGP may be able to substitute for TAP1/TAP2 in the course of assembly of Class I MHC molecules, then it is possible that increased PGP may reflect a compensatory change to permit continued MHC assembly in the face of poor TAP1/TAP2 performance. Alternately, increases in PGP function might be primary, and lead to a compensatory decline in TAP1/TAP2 activity. It would be of interest in this regard to see if overexpression of PGP might help to correct defects in antigen presentation and H-2 assembly in transporter-defective cell lines or knockout mice. Additional information on the molecular basis for overactivity of PGP in resting T cells of old mice may also provide insights into the differences in IL-2 responsiveness and functional abilities between the R123'° and R123'° T-cell subsets in young and old mice.

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