[^{14}C]doxorubicin in the absence or presence of 30 μmol/L nonradiolabeled TEA, mitoxantrone, or doxorubicin for 10 min. Uptake was terminated by adding excess ice-cold uptake buffer. Cells were washed thoroughly three times with 1 mL ice-cold uptake buffer and then lysed by alkalization. The cell lysates were transferred to scintillation vials containing scintillation fluid, and the radioactivities were measured in a liquid scintillation counter (LS 6000SE; Beckman Coulter). Cells washed with the uptake buffer immediately after addition of the assay mix were used as the zero-time point, representing nonspecific binding of the drug to the cells. A TEA uptake assay to select the clones expressing SLC22A4 (OCTN1) was done by incubating the cells with 60 μmol/L [^{14}C]TEA for 10 min.

Statistical Analysis for Drug Sensitivity Assay and Uptake Assay

Differences between the mean values were analyzed by two-sided Student's *t* test and results were considered statistically significant at $P < 0.05$.

Results

mRNA Expression of SLCO and SLC22 Family Members in Human Tissues

Figure 1A is a clustered image map ("heat map"; ref. 20) that displays the patterns of mRNA expression of the SLCO (11 genes) and SLC22 (17 genes) families across 29 human tissues (including fetal liver, brain, and kidney). Red and blue indicate high and low expression relative to the mean expression of a given transporter across all tissues, respectively (see also Supplementary Table S1). Subsets of the SLC genes expressed abundantly in the liver, brain, and kidney clustered on the heat map. For example, *SLCO1B1*, *SLCO1B3*, *SLC22A1*, *SLC22A7*, and *SLC22A9*, which are expressed at high levels in the liver, form a cluster. *SLC22A15*, *SLC22A17*, *SLCO1A2*, and *SLCO1C1*, expressed at high levels in tissues of the nervous system such as those of the brain and spinal cord, form another cluster. Whereas 9 of 17 SLC22 family genes were expressed at high levels in the kidney, of the SLCO family, only *SLCO4C1* was expressed at high levels in that tissue. Other than the liver, brain, and kidney, high levels of gene expression were observed in the testis, lung, mammary gland, and retina. These results are consistent with those reported in human subjects (6–8).

mRNA Expression in the NCI-60 Cancer Cell Line Panel

Figure 1B shows the mRNA expression of SLC across the NCI-60 cancer cell lines. The data indicate that SLC transporters are expressed at similar levels in normal tissues and across the cancer panel (Supplementary Table S2). In comparison with published expression patterns of gene expression (5, 19), the SLCO and SLC22 family members show less coherence. The distribution of SLC transporters on the gene dendrogram appears to be independent of sequence-homology categories. In our previous study on 48 ABC transporters, the pattern of expression correlated to some extent with tissue of origin of the cells. In particular, melanoma cells were found to express a characteristic set of

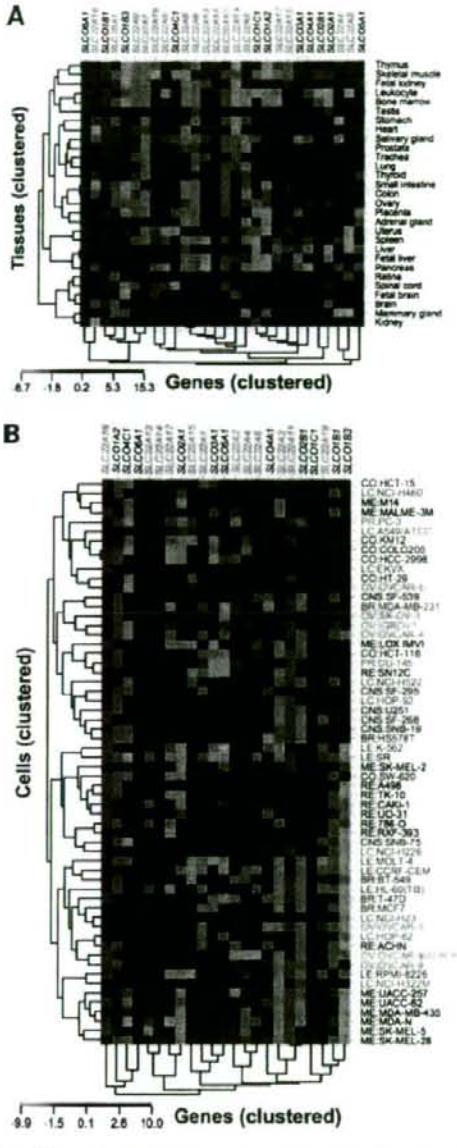

Figure 1. mRNA expression of SLCO and SLC22 family members in normal tissue samples (**A**) and the NCI-60 cancer cell line panel (**B**). These clustered image maps show patterns of gene expression assessed by real-time quantitative RT-PCR. *Red*, high expression; *blue*, low expression. The hierarchical clustering on each axis was done using the average linkage algorithm with 1 - *r* as the distance metric, where *r* is the Pearson's correlation coefficient, after subtracting column means. Included among the NCI-60 cancer cell lines are leukemias (*LE*), melanomas (*ME*), and cancers of breast (*BR*), central nervous system (*CNS*), colon (*CO*), lung (*LC*), ovarian (*OV*), prostate (*PR*), and renal (*RE*) origin. Three independent real-time quantitative RT-PCR measurements were done. For more details, see Supplementary Tables S1 and S2.

ABC transporters, but renal, central nervous system, and ovarian cancer cell lines also tended to cluster (5). The present study suggests that the relation of SLC transporter expression to tissues is less explicit, as only melanoma and renal cancer cell lines tended to form clusters. However, consistent with earlier molecular profiles, the two lumenal, hormone-dependent breast cancer lines, MCF7 and T47D, cluster together, and MDA-N, an ERBB2 transfectant of MDA-MB435 clusters with its parental line. Interestingly, *SLC22A6, SLC22A7, SLC22A8, SLC22A9*, and *SLC22A12* mRNAs were not detected in any of the 60 cancer cell lines, although they were found to be expressed in the human mammary (*SLC22A6, SLC22A7, SLC22A8*, and *SLC22A12*), kidney (*SLC22A6, SLC22A7, SLC22A8, SLC22A9*, and *SLC22A12*), liver (*SLC22A7, A9*), and retina (*SLC22A6* and *SLC22A8*) mRNA samples. The expression matrix of the 23 extant SLC transporters in the 60 cells is presented in Supplementary Table S2.

Identification of Candidate Uptake Transporters for Anticancer Drugs

We hypothesize that a strong correlation of a SLC transporter with the activity of a given compound is due to increased transporter-mediated cellular accumulation of the drug and that we can thus predict substrates of individual transporters through bioinformatic analysis. To verify this hypothesis, we correlated the expression patterns of SLC transporters with growth inhibition data for 1,429 compounds from the National Cancer Institute DTP. In particular, we were looking for positively correlated drug-gene pairs, which may indicate that a given compound can inhibit growth of the cancer cells more strongly if an SLC transporter is overexpressed. Using the expression data presented in Supplementary Table S2, we calculated the Pearson's correlation coefficient for each gene-drug pair.

Of the resulting 32,867 relationships (23 genes × 1,429 compounds), the correlation values showed a distribution of r values (Fig. 2) comparable with that of previous correlations for ABC transporters with the same compound subset (5). Our assumption was that the large majority of relationships would, in fact, be uncorrelated and this is indeed the case; 98.4 % of drug-gene correlations are $-0.4 < r < 0.4$. To narrow the list of correlated drug-gene pairs, we used both a simple Bonferroni procedure and the Benjamini-Hochberg False Discovery Rate procedure (21) to adjust for multiple testing of all 28 genes and all 1,429 compounds simultaneously. The analysis yielded several significantly positively correlated gene-drug pairs (at a false discovery rate < 0.1; see Supplementary Table S3). To verify that statistical correlations are based on a functional relationship [where an increase in the function of the gene product (uptake transporter) results in increased drug sensitivity], we performed follow-up experiments in the laboratory.

For our initial *in vitro* validation work, we chose to focus on SLC22A4, which was expressed at high levels in kidney, bone marrow, lung, prostate, spinal cord, and trachea human tissue samples. The number of drugs from the set of 1,429 that showed correlations of at least 0.4 with SLC22A4 was 18, where <1 would be expected in a set of 1,429 random correlations. Permutations of the cell lines showed that the mean number of correlations exceeding 0.4 among 1,429 correlations computed with permuted cell lines was 1.71. If we take 0.4 as a reasonable threshold for significance, then the Benjamini-Hochberg estimate of the proportion of false positives would be 1.71 / 18 < 0.1. An initial analysis had indicated that mitoxantrone (NSC301739) and doxorubicin (NSC123127), two anticancer drugs used widely in the anticancer regimens, show a highly significant Pearson correlation with the expression of the SLC22A4 (OCTN1) gene product (Fig. 3; Supplementary Table S3). The correlations of the GI_{50} values of mitoxantrone and doxorubicin with the $\log_2$ expression of SLC22A4 (OCTN1) were 0.45 ($P = 6.2 \times 10^{-5}$) and 0.43 ($P = 1.4 \times 10^{-4}$), respectively. Among the SLC genes examined in this study, these drugs revealed only one other (weaker) strong correlation with a second transporter; mitoxantrone with SLCO2A1 ($r = 0.35$) and doxorubicin with SLC22A5 ($r = 0.33$) (Supplementary Table S3).

Analysis of the SLC22A4 transporter drug-gene pairs revealed that 18 compounds gave a Pearson correlation coefficient ≥ 0.4. The NSC compounds along with their correlation coefficients, structure, common name(s), and correlations with other SLC transporters are given in Supplementary Table S6. Eleven of the 18 compounds are organic cations or formulated as acid salts (forming cations in solution), including 8 of the top 10 compounds, which strongly supports the notion that SLC22A4 is an organic cation transporter. We used statistical tools to prioritize gene-drug pairs for additional attention. In addition to the clinically used anticancer agents, we included two compounds that were predicted to be SLC22A4 substrates based

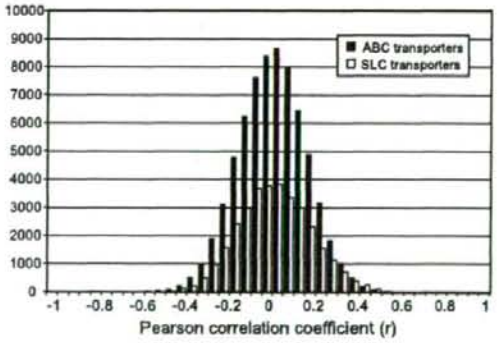

Figure 2. Distribution of r values for the SLC22 and SLCO drug-gene pairs (*white columns*). The distribution is overlaid with the r value distributions for the same compound drug-gene pairs set with ABC transporters (*gray columns*), showing the overall similarity in correlation distribution for the two transporter families. The Pearson correlation coefficient r values for ABC transporters are weighted slightly to the negative, as a negative r value is predictive for compounds that are substrates for the efflux transporters, whereas the SLC22/SLCO r values are skewed slightly to the positive as substrates of SLC22/SLCO transporters would have enhanced cellular accumulation and therefore increased cytotoxicity when a given SLC22/SLCO transporter is overexpressed.

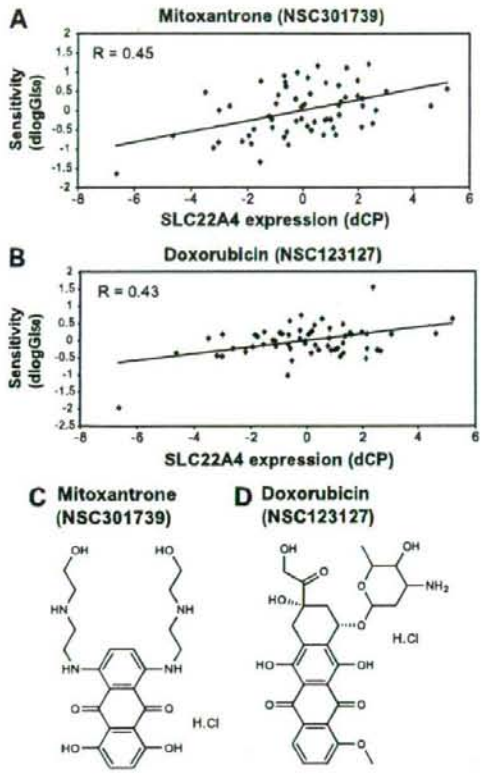

Figure 3. Prediction of substrates for a SLC [SLC22A4 (OCTN1)] from correlation analysis. Scatter plot showing the correlation of *SLC22A4* mRNA expression with sensitivity of the NCI-60 cancer cell lines to mitoxantrone (**A**) and doxorubicin (**B**). **C** and **D**, chemical structures of mitoxantrone and doxorubicin. (NSC numbers of drugs are shown in parentheses.) Both GI_{50} and CP values across the NCI-60 panel were mean-centered and multiplied by -1 (dlogGI_{50} and dCP, respectively) to indicate activity and expression relative to the mean.

on significant drug-gene correlation values. NSC59729 (sparsomycin; $r = 0.35$) and a structural analogue, NSC251819 ($r = 0.34$), were also validated in cytotoxicity assays. These two compounds both possess alkyl sulfoxy moieties similar to those of busulfan (NSC750) described above. Their structures are presented in Supplementary Fig. S1. (Drugs are designated by their National Service Center numbers in the DTP drug database).[9]

Establishment of Stable Transfectants of SLC22A4 (OCTN1)

We established stable transfectants of V5-tagged SLC22A4 (OCTN1) using KB-3-1 cell lines (derived from a single clone of human KB epidermoid carcinoma cells). KB-3-1 cells transfected with vector only plasmid were also established as a control (Mock/KB-3-1). The expression and function of SLC22A4 (OCTN1) in the stably transfected cells (SLC22A4 (OCTN1)/KB-3-1) were confirmed by a combination of real-time quantitative RT-PCR, Western blot analysis, immunocytochemical analysis, and accumulation assays. In the Western blot analysis, the protein was detected at approximately 62 kDa in two clones of SLC22A4 (OCTN1)/KB-3-1 (Fig. 4A). Immunocytochemical analysis using anti-V5-FITC antibody confirmed that the expressed protein was localized predominately in the plasma membrane (Fig. 4B). RT-PCR revealed the CP value (where a lower CP indicates greater expression) of SLC22A4 in the engineered SLC22A4/KB-3-1 cell line as 24.7. This expression is 5-fold greater than the highest expressing NCI-60 cell line, NCI-H226 (CP = 27.17) and 9-fold greater than the highest tissue expression; kidney (CP = 27.9). In the uptake assay, the accumulation level of a known SLC22A4 (OCTN1) substrate, [^{14}C]TEA, was 7.2-fold higher in SLC22A4 (OCTN1)/KB-3-1 cells than in Mock/KB-3-1 cells (Fig. 4C), indicating that the established cell lines express high levels of functionally competent SLC22A4 protein.

Sensitivity of SLC22A4 (OCTN1) KB-3-1 Cells to Mitoxantrone and Doxorubicin

To test whether SLC22A4 (OCTN1) sensitizes the cells to mitoxantrone and doxorubicin treatment as predicted by the bioinformatic analysis, we compared SLC22A4 (OCTN1)/KB-3-1 with Mock/KB-3-1 in drug sensitivity assays (Fig. 5). The IC_{50} values of mitoxantrone and doxorubicin in SLC22A4 (OCTN1)/KB-3-1 after 72-h drug exposure were 4.6- and 3.6-fold lower (indicating greater sensitivity) than those in Mock/KB-3-1, respectively (Table 1). In experiments not documented here, we also tested NSC59729 and NSC251819 and found that SLC22A4 (OCTN1)/KB-3-1 was 2.1- and 2.6-fold more sensitive to the two compounds, respectively, than was Mock/KB-3-1 ($P < 0.05$). Cisplatin, a compound not predicted to be toxic to SLC22A4-expressing cells, showed equivalent cytotoxicity to both mock and transporter-expressing cell lines (data not shown).

Uptake of Mitoxantrone and Doxorubicin by SLC22A4 (OCTN1) KB-3-1 Cells

Results obtained with the cytotoxicity assays indicated that the presence of SLC22A4 sensitizes cells. To test if the increased sensitivity of the cells could be explained by an increased uptake of the compounds, we performed accumulation experiments with radiolabeled mitoxantrone and doxorubicin. Cells were incubated with 3 μmol/L [^{3}H]mitoxantrone or [^{14}C]doxorubicin at 37°C and reactions were stopped at designated time points by adding excess ice-cold uptake buffer. As shown in Fig. 6A and B, uptake of both drugs was significantly increased in SLC22A4 (OCTN1)/KB-3-1 cells compared with Mock/KB-3-1 cells. Addition of nonradiolabeled 30 μmol/L TEA (a known substrate of SLC22A4) to the reaction buffer resulted in a decrease in the accumulation level of [^{3}H]mitoxantrone and [^{14}C]doxorubicin in SLC22A4 (OCTN1)/KB-3-1 cells, with little effect in Mock/KB-3-1 cells (Fig. 6C and D), consistent

[9] Information on National Service Center compound is available at http://spheroid.ncifcrt.gov/spheroid/.

with the notion that TEA and mitoxantrone/doxorubicin are competing for a common transport mechanism.

Discussion

Compared with efflux transporters (ABC transporters that are considered targets to overcome MDR; ref. 4), relatively little attention has been paid to uptake transporters in cancer research. However, considering that most anticancer drugs need to first enter cancer cells and accumulate to be effective, uptake transporters are expected to play a critical role in ensuring drug efficacy (22). There is already evidence that lowered expression of the copper influx transporter CTR1 (SLC31A1) is implicated in the development of cellular resistance to the cancer drug cisplatin (23), which is a substrate for the nutrient transporter (24).

The NCI-60 cancer cell lines have been more extensively profiled at the DNA, mRNA, protein, and functional levels than any other set of cell lines in existence (see http://dtp.nci.nih.gov/ and http://discover.nci.nih.gov/cellminer). In addition, patterns of drug activity across the cell lines and patterns of cell sensitivity across the set of tested drugs have been shown to contain detailed information on mechanisms of action and resistance (25, 26). The NCI-60 database has been successfully exploited to identify ABC transporters that confer MDR and compounds whose activity is decreased or potentiated by them (5, 27, 28). Using the same pharmacogenomic approach, identification of transporters that mediate the uptake of compounds should also be possible. Previous transcript expression profiling of the NCI-60 using cDNA arrays of >9,000 elements (29), Affymetrix Hu6800 oligonucleotide chips (19), or a dedicated platform representing the "transportome" (16) proved useful for identifying molecular biomarkers of chemoresistance. Our aim was to generate more reliable expression data for the SLC22 and SLCO genes to identify functionally relevant drug-gene pairs. Microarray studies are restricted by the limitations of the technology (such as low coverage or low sensitivity). Our earlier meta-analysis of the Staunton et al. (29) and Scherf et al. (19) arrays revealed that the data sets generated by the two platforms show very poor correlation (30). Combined, these two arrays contain only 8 of the 23 genes analyzed in our study (those with detectable expression); the Staunton cDNA array examined seven SLC transporters that are also reported in our study (SLC22A3, SLC22A4, SLC22A15, SLC22A17, SLCO1A2, SLCO1B1, and SLCO2B1), the strongest correlation between the two platforms being that of SLC22A3 (r = 0.43). The remaining Pearson correlation values (r) range from -0.5 to 0.29, revealing little consensus between the common genes in these two datasets. The Scherf Affymetrix data reported only two genes common to our RT-PCR data (SLCO1A2 r = 0.00 and SLCO2A1 r = 0.09) with no correlation evident between the two sets of genes.

We chose to measure transcript expression by real-time quantitative RT-PCR. Although PCR is a low-throughput (and necessarily labor-intensive) technology, the number of genes of interest here did not warrant a more expensive high-throughput approach. A normalizing gene was not employed, as previous experience while measuring ABC transporter expression by RT-PCR showed high variability in the expression of five control genes, and mean expression normalization had reliably predicted drug-gene pairs of known ABC transporter substrates, and new substrates and selective compounds that were validated *in vitro* (5). We focused our attention on highly significant positive correlations between transporters and individual compounds, with an emphasis on clinically relevant drugs.

The number of positive correlations was similar in the case of SLC transporters (153 drug-gene pairs with $r \geq 0.50$) and ABC transporters (137 drug-gene pairs with $r \geq 0.50$). As there were fewer total SLC drug-gene pairs in this study, this indicates that 0.47% of compounds were positively correlated with the expression of a SLC transporter, an increase over the 0.20% discovery rate in the ABC transporter study (5). A positively correlated drug-gene pair indicates that increased expression of the gene is related

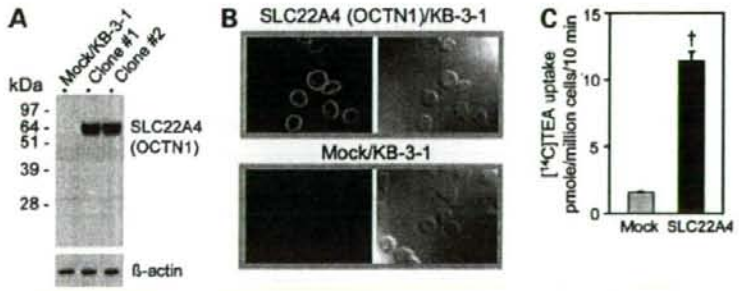

Figure 4. Confirmation of protein expression and function of SLC22A4 (OCTN1) in established stable transfectants used in all follow-up experiments to evaluate whether correlations reveal functional interaction. **A,** crude membrane fraction from cells stably transfected with mock vector (Mock/KB-3-1) or SLC22A4 (OCTN1) was subjected to Western blot analysis with anti-V5-HRP antibody. Two clones (clones #1 and #2) of SLC22A4 (OCTN1)/KB-3-1 in which protein expression was confirmed are shown. **B,** cellular localization of SLC22A4 (OCTN1) in the SLC22A4 (OCTN1)/KB-3-1 and Mock/KB-3-1 cells was assessed by immunocytochemical analysis using anti-V5-FITC antibody. Result of SLC22A4 (OCTN1)/KB-3-1 clone #1 is shown as a representative. **C,** uptake of a known substrate ([^{14}C]TEA, 60 μmol/L) for SLC22A4 (OCTN1) by SLC22A4 (OCTN1)/KB-3-1 and Mock/KB-3-1 cells. †, $P < 0.005$, Mock/KB-3-1 versus SLC22A4 (OCTN1)/KB-3-1.

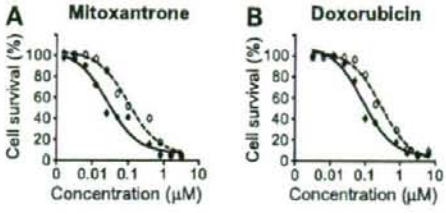

Figure 5. Validation of the prediction by drug sensitivity assay. Growth inhibition of the SLC22A4 (OCTN1)/KB-3-1 (♦) and Mock/KB-3-1 (○) cells treated with either mitoxantrone (**A**) or doxorubicin (**B**) for 72 h was evaluated by CCK-8 assay. Result of SLC22A4 (OCTN1)/KB-3-1 clone #1 is shown as a representative. Each experiment was done independently at least three times. Note that error bars are mostly obscured by the data points and that differences are statistically significant (as shown in Table 1).

to increased growth-inhibitory activity. In the case of ABC efflux transporters, the mechanism by which the toxicity of positively correlated compounds such as NSC73306 is potentiated by P-glycoprotein is unclear (27, 31). The higher ratio of positively correlated drug-gene pairs in the SLC-set is in accord with the function of these transporters as facilitators of influx (Fig. 2).

In contrast to the previous drug-gene correlations observed with ABC transporters (using the same drug activity dataset), the number of significant negative correlations was less pronounced. Of the 68,592 relationships (48 genes × 1,429 compounds) calculated for ABC transporters, 48 drug-gene pairs revealed significant inverse correlations ($r < -0.55$), suggestive of a transporter-substrate relationship in which the transporter protects the cells by keeping the recognized substrates below a cell-killing threshold. In contrast, only two drug-gene pairs ($r < -0.55$) with SLC transporters showed such a strong inverse correlation to any of the drugs analyzed—the napthalendiones NSC623758 (SLCO2B1 $r = -0.65$) and NSC618315 (SLC22A18 $r = -0.6$). This low number of negative correlations (relative to drug-gene pairs observed among the ABC transporters) is entirely logical given that the SLC families are recognized as importers and as such are not hypothesized to play a role in actively lowering cellular accumulation (Fig. 2).

It is not clear how coexpression of influx and efflux transporters that are diametrically opposed to one another in function would affect cytotoxicity (which is tied in most cases to cellular accumulation) and therefore impinge on correlations between drug activity and SLC22/SLCO expression. Despite the considerable overlap in the substrate specificity of organic transporters and members of the ABC transporter family, the bioinformatics analysis has successfully identified mitoxantrone and doxorubicin as SLC22A4 substrates. Correlations between SLC22A4 expression for each of the three best-characterized MDR efflux transporters revealed that there is not a strong correlation between SLC22A4 and ABCB1 (P-glycoprotein $r = -0.21$), ABCC1 (MRP1 $r = -0.10$), and ABCG2 ($r = -0.09$) expression. The three strongest ABCB1-expressing cell lines do express lower than average levels of SLC22A4: NCI-ADR-RES (ABCB1 = 12.28 and SLC22A4 = −6.62), HCT-15 (ABCB1 = 10.08 and SLC22A4 = −1.83), and UO-31 (ABCB1 = 4.68 and -SLC22A4 = −1.33). Grube et al. have examined the expression of a SLC22A5/ABCB1 double-transfection cell line and showed that the cell line dramatically increased transcellular transport, but not accumulation, suggesting that ABCB1 expression could negatively affect the strength of correlations. Yet the majority of NCI-60 cell lines do not express high levels of ABCB1, and removing NCI-ADR-RES, HCT-15, and UO-31 cell lines from the correlation analysis of SLC22A4 and ABCB1 reveals no correlation at all between their gene expression patterns ($r = -0.01$).

There is strong structural consistency among the 18 compounds that correlated with SLC22A4 expression. All but one of the 18 compounds contains at its core three or four fused rings with a high degree of aromaticity, the exception being NSC750, the alkylating agent busulfan (Supplementary Table S6). Six of these compounds are highly structurally related anthracycline derivatives: NSC123127 (doxorubicin, Adriamycin), NSC354646, NSC357704, NSC275647, and NSC164011 (rubidazone, zorubicin), the structural variation being at either the terminus of the side chain or substitution on the primary amine of the daunosamine sugar (32). The anthracycline daunorubicin (NSC82115) also appeared in the 50 most correlated compounds with SLC22A4 expression ($r = 0.35$). Five additional compounds, mitoxantrone (NSC301739), piroxantrone (NSC349174),

Table 1. IC_{50} values of mitoxantrone (NSC301739), doxorubicin (NSC123127), sparsomycin (NSC59729), and NSC251819 in the SLC22A4 (OCTN1)/KB-3-1 and Mock/KB-3-1 cells

	IC_{50}		Sensitivity factor*
	Mock/KB-3-1	SLC22A4/KB-3-1	
Mitoxantrone (NSC301739; nmol/L)	12.69 ± 1.71	2.75 ± 0.16	4.6
Doxorubicin (NSC123127; nmol/L)	33.36 ± 3.37	9.31 ± 0.11	3.6
Sparsomycin (NSC59729; mmol/L)	0.59 ± 0.13	0.28 ± 0.09	2.1
NSC251819 (mmol/L)	15.75 ± 3.98	6.06 ± 0.62	2.6

*The sensitivity factor is defined as the ratio of the mean IC_{50} in the Mock/KB-3-1 cells to that in the SLC22A4 (OCTN1)/KB-3-1 cells. Mean ± SD from three to four independent experiments, each done in triplicate. $P < 0.01$, Mock/KB-3-1 versus SLC22A4 (OCTN1)/KB-3-1.

NSC355644, NSC693120, and NSC625530 are derived from anthracenediones; and two closely related compounds possess structural components of etoposide (NSC668380 and NSC644945; ref. 33).

It is known that a given compound may be a low or high specificity substrate for multiple SLC transporters (see, for example, the SLC tables at http://www.bioparadigms.org/slc/intro.asp). To ascertain whether there appeared to be functional overlap among the compounds that correlated with SLC22A4, we sought compounds with a Pearson correlation coefficient > 0.30. Thirteen of 18 compounds returned a positive correlation with one or more other SLCO or SLC22 genes (6 correlated with two or more alternate SLC genes) and remarkably the eicosanoid transporter SLCO2A1 (8 compounds) and the polyspecific cation transporter SLC22A5 (6 compounds) showed multiple common correlations (Supplementary Table S6) suggestive of cross-recognition among SLC homologues. SLC22A5 (OCTN2) has been shown to be expressed in human heart endothelial cells (34), and whereas SLC22A4 expression in the human heart has not been reported previously, both transporters show similar mean-centered mRNA expression in this study (Fig. 1; Supplementary Table S1; SLC22A4 = 0.52 and SLC22A5 = 0.82). It may be that SLC-facilitated uptake of the anthracyclines and anthracenediones described above is in part responsible for the clinically limiting cardiotoxicity of these agents (32). Furthermore, the degeneracy in substrate recognition among SLC transporters would result in a number of lower correlation coefficients for a compound (as is seen in our study), rather than the strong (negative) correlation coefficients for efflux substrates of the ABC transporters, as described above.

Among the top-scoring correlations, we were interested in the SLC22A4 (OCTN1)-mitoxantrone and SLC22A4 (OCTN1)-doxorubicin pairs. SLC22A4 (OCTN1) has been characterized as a low-affinity carnitine transporter (35) and is best known for its association with Crohn's disease (36). Although some members of the SLCO and SLC22 family such as *SLC22A15* have been implicated in carcinogenesis (37), SLC22A4 (OCTN1) expression has not been linked to cancer. We found *SLC22A4* to be expressed abundantly in the 60 cancer cell lines that originate from a variety of different tissues, such as lung (NCI-H226, EKVX, NCI-H322M, and NCI-H460), melanoma (LOX IMVI), central nervous system (SF-295), kidney (A498 and UO-31), and breast (HS578T). Among the 60 cell lines, only OVCAR-8/ADR-RES (38), which overexpresses *MDR1* and is highly resistant to doxorubicin (Adriamycin), did not express *SLC22A4*.

Currently, there are no data to suggest the involvement of SLC22A4 in pharmacologic response, yet it cannot be ruled out that it affects cell survival by modulating the electrochemical gradient or by altering the uptake of physiologic compounds and toxins. To test whether the observed correlations reflect an actual ability of the protein to confer sensitivity, we established stable transfectants of SLC22A4 (OCTN1) and characterized their phenotype in drug sensitivity assays. Expression of SLC22A4 (OCTN1) resulted in heightened sensitivity to both mitoxantrone and doxorubicin. Although the levels of increased sensitivity were relatively low, ample evidence indicates that even small (2- to 4-fold) changes in drug sensitivity can have a significant effect on the clinical efficacy of anticancer treatment (2). For many anticancer drugs, toxic-to-therapeutic ratios are low; therefore, even a small change in drug sensitivity can hamper chemotherapy. Expression of SLC22A4 (OCTN1) also resulted in increased cellular uptake of mitoxantrone and doxorubicin. Presumably,

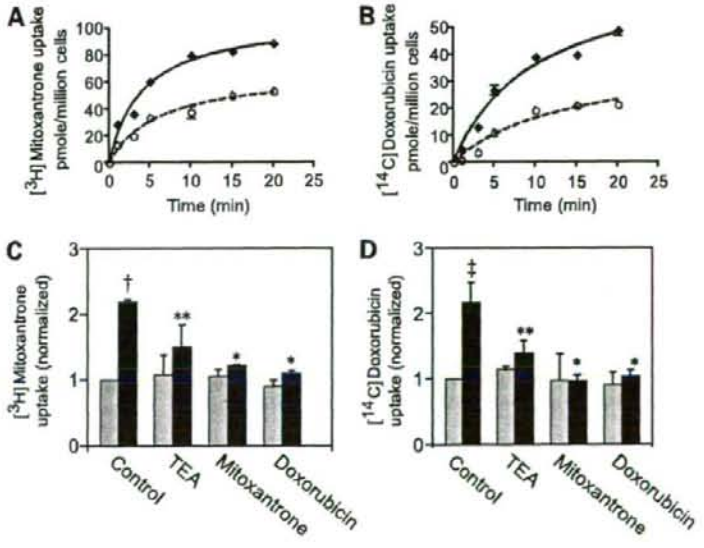

Figure 6. Uptake of mitoxantrone and doxorubicin by the SLC22A4 (OCTN1)/KB-3-1 and the Mock/KB-3-1 cells. **A** and **B**, time course of uptake of [^{3}H]mitoxantrone (3 μmol/L) and [^{14}C]doxorubicin (3 μmol/L) by the SLC22A4 (OCTN1)/KB-3-1 (♦) and Mock/KB-3-1 (○) cells. **C** and **D**, uptake of [^{3}H]mitoxantrone (3 μmol/L) and [^{14}C]doxorubicin (3 μmol/L) by the SLC22A4 (OCTN1)/KB-3-1 (*black columns*) and the Mock/KB-3-1 cells (*gray columns*) in the absence (control) or presence of 30 μmol/L nonradiolabeled TEA, mitoxantrone, or doxorubicin. Uptake values are shown in fold change to that of the Mock/KB-3-1 cells incubated in the absence of nonradiolabeled drugs. Mean ± SD from three independent experiments, each done in duplicate. †, $P < 0.005$; ‡, $P < 0.05$, SLC22A4 (OCTN1)/KB-3-1 versus Mock/KB-3-1 without nonradiolabeled compounds. *, $P < 0.005$; **, $P < 0.05$, SLC22A4 (OCTN1)/KB-3-1 without nonradiolabeled compounds versus SLC22A4 (OCTN1)/KB-3-1 with nonradiolabeled compounds.

SLC22A4 (OCTN1) mediates uptake of the weak base compounds, most of which bear positive charges within the physiologic pH range and also in the acidic conditions characteristic of the tumor microenvironment (39).

Our bioinformatics results and *in vitro* experiments imply that uptake transporters can facilitate uptake of hydrophobic drugs that have charged forms and increase total uptake of those drugs even in the presence of a background level of diffusion across the plasma membrane. This is in contrast to Grigat et al., who used competition assays with ergothionein against verapamil and several noncytotoxic histamine antagonists to conclude that SLC22A4 is not a "multispecific" cationic drug transporter (40). Because gene expression levels of SLC22A4 (OCTN1) in our transfectants are comparable with those in some normal tissues (e.g., kidney) and cancers, physiologic amounts of SLC22A4 (OCTN1) may determine the sensitivity of the cell to drugs such as mitoxantrone and doxorubicin. Thus, both effectiveness of anticancer drugs to treat cancer and the toxicity of these drugs in normal tissues may be determined partially by levels of that transporter (and, by extension, other SLC22 and SLCO transporters).

Expression of SLC22A4 (OCTN1) also made cells more sensitive to two other compounds, NSC59729 and NSC251819, which were predicted to be substrates for SLC22A4 (OCTN1) from the correlation analysis (Supplementary Table S3; Table 1).[10] Overall, these results support the idea that correlation of the gene expression of the SLCO and SLC22 families with activity patterns in the 60 cell lines can be used to identify their potential substrates among the >100,000 compounds tested by the National Cancer Institute. The correlation data could potentially provide information on significant relationships between drugs and uptake transporters to guide the development of transporter-targeted chemotherapy.

It should be noted that the correlative approach described here has statistical limitations: the sample size, the experimental uncertainty, and the fact that multiple tests of significance are being done simultaneously (hence, the "multiple testing" corrections we describe in the text and in Materials and Methods). There are also biological limitations: (*a*) as is the case for almost all transcript profiling studies, there remains uncertainty about the relationship between mRNA and protein expression and the relationship of both to function; (*b*) correlation does not imply causality; and (*c*) the potential for cross-recognition of substrates would be expected to weaken strong drug-gene correlations. The correlated drug-gene pairs shown in Supplementary Table S3 serve as a starting point for the generation of additional hypotheses. The results presented here indicate that (*a*) several compounds (drugs and drug candidates) inhibit the growth of cancer cells more strongly if a SLC transporter is overexpressed and (*b*) SLC transporters can play a decisive role in the chemosensitivity of cancer cells. Additional follow-up experiments are under way to identify further SLC transporters that may influence chemosensitivity.

[10] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the staff of the National Cancer Institute DTP for providing the total RNA of the NCI-60 cancer cell lines and generation of the pharmacologic database used in this study and George Leiman for editorial assistance.

References

1. Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med 2002;53:615–27.

2. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002;2:48–58.

3. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 1999;39:361–98.

4. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006;5:219–34.

5. Szakacs G, Annereau JP, Lababidi S, et al. Predicting drug sensitivity and resistance: profiling ABC transporter genes in cancer cells. Cancer Cell 2004;6:129–37.

6. Mikkaichi T, Suzuki T, Tanemoto M, Ito S, Abe T. The organic anion transporter (OATP) family. Drug Metab Pharmacokinet 2004;19:171–9.

7. Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. Pflugers Arch 2004;447:653–65.

8. Koepsell H, Endou H. The SLC22 drug transporter family. Pflugers Arch 2004;447:666–76.

9. Abe T, Unno M, Onogawa T, et al. LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. Gastroenterology 2001;120:1689–99.

10. Okabe M, Unno M, Harigae H, et al. Characterization of the organic cation transporter SLC22A16: a doxorubicin importer. Biochem Biophys Res Commun 2005;333:754–62.

11. Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood 2004;104:3739–45.

12. Ciarimboli G, Ludwig T, Lang D, et al. Cisplatin nephrotoxicity is critically mediated via the human organic cation transporter 2. Am J Pathol 2005;167:1477–84.

13. Yonezawa A, Masuda S, Yokoo S, Katsura T, Inui K. Cisplatin and oxaliplatin, but not carboplatin and nedaplatin, are substrates for human organic cation transporters (SLC22A1-3 and multidrug and toxin extrusion family). J Pharmacol Exp Ther 2006;319:879–86.

14. Zhang S, Lovejoy KS, Shima JE, et al. Organic cation transporters are determinants of oxaliplatin cytotoxicity. Cancer Res 2006;66:8847–57.

15. Hall MD, Okabe M, Shen DW, Liang XJ, Gottesman MM. The role of cellular accumulation in determining sensitivity to platinum-based chemotherapy. Annu Rev Pharmacol Toxicol 2008;48:495–535.

16. Huang Y, Anderle P, Bussey KJ, et al. Membrane transporters and channels: role of the transportome in cancer .chemosensitivity and chemoresistance. Cancer Res 2004;64:4294–301.

17. Weinstein JN. Spotlight on molecular profiling: "integromic" analysis of the NCI-60 cancer cell lines. Mol Cancer Ther 2006;5:2601–5.

18. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:RESEARCH0034.

19. Scherf U, Ross DT, Waltham M, et al. A gene expression database for the molecular pharmacology of cancer. Nat Genet 2000;24:236–44.

20. Weinstein JN, Myers TG, O'Connor PM, et al. An information-intensive approach to the molecular pharmacology of cancer. Science 1997;275:343–9.

21. Reiner A, Yekutieli D, Benjamini Y. Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics 2003;19:368–75.

22. Huang Y, Sadee W. Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells. Cancer Lett 2006;239:168–82.

23. Song IS, Savaraj N, Siddik ZH, et al. Role of human copper transporter Ctr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and cisplatin-resistant cells. Mol Cancer Ther 2004;3:1543–9.

24. Lin X, Okuda T, Holzer A, Howell SB. The copper transporter CTR1 regulates cisplatin uptake in *Saccharomyces cerevisiae*. Mol Pharmacol 2002;62:1154–9.

25. Paull KD, Shoemaker RH, Hodes L, et al. Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm. J Natl Cancer Inst 1989;81:1088–92.

26. Weinstein JN, Kohn KW, Grever MR, et al. Neural computing in cancer drug development: predicting mechanism of action. Science 1992;258:447–51.

27. Ludwig JA, Szakacs G, Martin SE, et al. Selective toxicity of NSC73306 in MDR1-positive cells as a new strategy to circumvent multidrug resistance in cancer. Cancer Res 2006;66:4808–15.

28. Alvarez M, Paull K, Monks A, et al. Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein in the cell lines of the National Cancer Institute Anticancer Drug Screen. J Clin Invest 1995;95:2205–14.

29. Staunton JE, Slonim DK, Coller HA, et al. Chemosensitivity prediction by transcriptional profiling. Proc Natl Acad Sci U S A 2001;98:10787–92.

30. Lee JK, Bussey KJ, Gwadry FG, et al. Comparing cDNA and oligonucleotide array data: concordance of gene expression across platforms for the NCI-60 cancer cells. Genome Biol 2003;4:R82.

31. Wu C-P, Shukla S, Calcagno AM, Hall MD, Gottesman MM, Ambudkar SV. Evidence for dual mode of action of a thiosemicarbazone, NSC73306: a potent substrate of the multidrug resistance-linked ABCG2 transporter. Mol Cancer Ther 2007;6:3287–96.

32. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 2004;56:185–229.

33. Meresse P, Dechaux E, Monneret C, Bertounesque E. Etoposide: discovery and medicinal chemistry. Curr Med Chem 2004;11:2443–66.

34. Grube M, Meyer zu Schwabedissen HE, Prager D, et al. Uptake of cardiovascular drugs into the human heart: expression, regulation, and function of the carnitine transporter OCTN2 (SLC22A5). Circulation 2006;113:1114–22.

35. Tamai I, Yabuuchi H, Nezu J, et al. Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. FEBS Lett 1997;419:107–11.

36. Peltekova VD, Wintle RF, Rubin LA, et al. Functional variants of OCTN cation transporter genes are associated with Crohn disease. Nat Genet 2004;36:471–5.

37. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. Science 2007;318:1108–13.

38. Bussey KJ, Chin K, Lababidi S, et al. Integrating data on DNA copy number with gene expression levels and drug sensitivities in the NCI-60 cell line panel. Mol Cancer Ther 2006;5:853–67.

39. Minchinton AI, Tannock IF. Drug penetration in solid tumours. Nat Rev Cancer 2006;6:583–92.

40. Grigat S, Harlfinger S, Pal S, et al. Probing the substrate specificity of the ergothioneine transporter with methimazole, hercynine, and organic cations. Biochem Pharmacol 2007;74:309–16.

J Appl Physiol 104: 1690–1696, 2008
First published April 10, 2008; doi:10.1152/japplphysiol.01044.2007

Exercise training enhances in vivo tuberculosis purified protein derivative response in the elderly

Mitsuharu Okutsu,[1] Yuko Yoshida,[2] Xiumin Zhang,[3] Akira Tamagawa,[3] Takayoshi Ohkubo,[4] Ichiro Tsuji,[5] and Ryoichi Nagatomi[3]

[1]*Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo,* [2]*Research Team for Promoting Independence of the Elderly, Tokyo Metropolitan Institute of Gerontology, Tokyo,* [3]*Medicine and Science in Sports and Exercise, Tohoku University Graduate School of Medicine, Sendai,* [4]*Clinical Pharmacology and Therapeutics, Tohoku University Graduate School of Pharmaceutical Sciences, Sendai, and* [5]*Epidemiology, Department of Public Health and Forensic Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan*

Submitted 30 September 2007; accepted in final form 25 March 2008

Okutsu M, Yoshida Y, Zhang X, Tamagawa A, Ohkubo T, Tsuji I, Nagatomi R. Exercise training enhances in vivo tuberculosis purified protein derivative response in the elderly. *J Appl Physiol* 104: 1690–1696, 2008. First published April 10, 2008; doi:10.1152/japplphysiol.01044.2007.—We investigated the effect of 25 wk of exercise training on in vivo immune measures that depend on T helper 1 (Th1) and T helper 2 (Th2) immune responses in the elderly as a substudy of a randomized controlled trial to investigate health benefits of regular exercise training for the elderly. Sixty-five healthy elderly volunteers were randomly assigned to either an exercise training group ($n = 32$) or a sedentary control group ($n = 33$). The area of skin reaction to purified protein derivative (PPD) of tuberculin that depends on Th1 activation and the concentrations of serum IgG subclasses and IgE were evaluated before and after 25-wk intervention. All participants completed 25 wk of training. Thirty-one participants of the exercise group and all control group underwent immunological analyses, but only 30 from the exercise group and 21 from the control group had the PPD skin reaction assessment. Repeated-measures ANOVA revealed a significant interaction between time and exercise intervention, which appeared as an enhanced skin reaction to tuberculin PPD ($P < 0.05$) and a reduced serum IgG4 concentration, the production of which depends on Th2-dependent class switching ($P < 0.05$), in the exercise group after 25 wk. No immune variables changed in the control group. These result supports the hypothesis that exercise training favors in vivo Th1 immune response in elderly persons.

delayed-type hypersensitivity; T helper 1 cells; immunoglobulin G4

T-CELL FUNCTIONS SUCH AS CYTOKINE production and cytotoxic effector functions in antigen recognition are essential for human adaptive immune defense and are subject to immune senescence. Mitogen-induced IL-2 and IFN-γ synthesis and T-cell proliferation decline with age (7). Elderly people generally exhibit significantly lower $CD8^+$ cytotoxic T-cell responses to mitogenic stimuli compared with younger people (3).

T helper cells with regulatory roles are subclassified into several distinct populations by their cytokine production patterns. T helper 1 (Th1) and T helper 2 (Th2) cells are important because they support two major aspects of the adaptive immune responses. Th1 cytokines, such as IL-2 and IFN-γ, support cell-mediated inflammatory reactions by activating cytotoxic and inflammatory functions, and Th2 cytokines, such as IL-4, IL-5, IL-6, IL-10, and IL-13, support humoral immune responses (19, 20). Th1 and Th2 cytokines are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype (8, 20). IFN-γ selectively inhibits proliferation of Th2 cells, and IL-4 and IL-10 inhibit cytokine synthesis by Th1 cells (20). This cross-regulation strongly biases the Th1 vs. Th2 responses during many infections in mice and humans (20).

Th1 clones are shown to induce delayed-type hypersensitivity (DTH) skin reactions (4), and IFN-γ is commonly expressed at sites of DTH reactions (10), whereas IgG4 and IgE production are known to be dependent on Th2-supported class switching. IgE production in allergic responses is strongly associated with Th2 activation (17, 23, 25).

A number of studies show that physical exercise affects Th1 and Th2 immune responses and the balance between these responses. Strenuous exercise decreases the percentage of circulating Th1 lymphocytes (28). Acute exhaustive exercise or short-term intense training significantly reduces the percentage and number of circulating Th1 cells but not of Th2 cells in endurance-trained cyclists (16). If the reduction in Th1 cytokine-releasing cells is associated with an attenuation in the Th1 immune response, such a reduction in frail elderly people may increase the risk of infection. There is, however, little evidence that exercise in apparently healthy elderly people increases their risk of infection. Rather, it is commonly believed that regular moderate exercise is beneficial. Our hypothesis is that the exercise regimens commonly employed for the elderly to maintain their physical functions and cardiovascular fitness may improve the T helper immune responses and perhaps potentiate Th1 immune response.

The aim of this study was to determine whether health-oriented exercise training employed to improve cardiovascular fitness would also maintain or potentiate Th1 immune responses in healthy elderly people. We tested our hypothesis in a substudy of a randomized controlled trial of a 25-wk exercise program in elderly people (Sendai Silver Center Trial), in which improvements in cardiovascular fitness and increased daily energy expenditure occurred in the ET group have been demonstrated (11, 29). Because Nakayama et al. (21) reported that nonresponders to the tuberculin PPD skin test, who had fewer circulating $CD4^+$ T lymphocytes and Th1 cells than

Address for reprint requests and other correspondence: R. Nagatomi, Medicine and Science in Sports and Exercise, Tohoku Univ. Graduate School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai 980-8575, Japan (e-mail: nagatomi@mail.tains.tohoku.ac.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

responders among immobile elderly subjects, had a higher risk of developing pneumonia, the tuberculin PPD skin reaction was employed as a clinically relevant measure associated with Th1 response. Because the tuberculin PPD response is antigen specific and may not reflect a general trend of Th1 and Th2 immune responses, serum IgG subclass concentration was measured, because Th1 immunity is known to be connected to the IgG1 and IgG3 subclasses (2, 22, 27, 30) and Th2 immunity to the IgG4 isotype and IgE (17). Therefore, the skin DTH reaction to tuberculin PPD, which is associated with IFN-γ producing Th1 activity, and serum level of IgG4, which is produced depending on Th2 activation, were evaluated before and after the 25-wk supervised exercise program.

MATERIALS AND METHODS

Design. This is a subsidiary study of the Sendai Silver Center Trial. Sendai Silver Center Trial was a randomized controlled trial in which health benefits of regular exercise training for the elderly was investigated (29). The protocol of the present study was approved by the Exercise Board of the Sendai Health and Welfare Foundation.

Study participants. Full details of selection criteria for the participants are in the previous report (29). We recruited through municipal advertisements independently living men and women aged 60 yr or older who resided in Sendai City. A total of 209 potential participants applied and attended the screening session. Of these, 121 were excluded for one or more of the following reasons: *1*) moderate to severe motor impairment or neurological deficits; *2*) a history of coronary heart disease within 6 mo of the study; *3*) patients with hypertension (systolic blood pressure >160 mmHg or diastolic blood pressure >100 mmHg); *4*) joint pain or arthritis limiting the full range of motion at shoulder, elbow, hip, or knee joints; *5*) mental or other conditions that could interfere with participation; *6*) other chronic disease that could interfere with participation; *7*) history of fracture of a lower extremity or injurious falls within 6 mo of the study; or *8*) use of antihypertensive agents, antiarrhythmic agents, nitroglycerides, digitalis, or vitamin supplements. Of the 88 remaining applicants, 78 attended the baseline measurement examination. A further 12 were excluded because of abnormal exercise electrocardiograms. The remaining 65 were grouped for age and sex and randomly allocated into either the exercise training group (ET, $n = 32$) or control group (C, $n = 33$) by epidemiologists who were masked from the measurement results (Fig. 1). All participants gave written informed consent. Baseline age, female sex, height, weight, and maximum oxygen consumption ($\dot{V}O_{2\,max}$) were not significantly different between ET and C groups (Table 1).

Two participants from the ET group and 12 participants from the C group did not agree to undergo tuberculin PPD examination for nonmedical, personal reasons. There were no statistical differences in all the baseline parameters between subjects who refused tuberculin PPD and subjects who agreed tuberculin PPD, as well as between the C group and ET group subjects who agreed to undergo tuberculin PPD responses.

Because steroidal and nonsteroidal anti-inflammatory medication or aspirin may largely affect the result of this study, those who took such medication throughout the study were to be excluded from the PPD examination. Medication status was checked every week on the occasion of exercise participation and monthly C group classes throughout the study.

ET group. We gave three 2-h classes per week for 25 wk. The participants in the ET group were asked to attend the classes at the Center at least twice a week. Each class started with a 30 min warm-up session, including static stretching of extremities and trunk muscles followed by a 10-min low-intensity stepping exercise. The main session consisted of an endurance exercise session using a bicycle ergometer and a resistance exercise session using rubber films.

Endurance exercise session. Participants cycled at 50–60 revolution/min on a bicycle ergometer at an individually prescribed intensity for 10–25 min. (Table 2). The intensity of exercise was determined according to the guidelines for exercise prescription of American College of Sports Medicine. The intensity of exercise was calculated as a percentage of heart rate reserve, based on the participant's

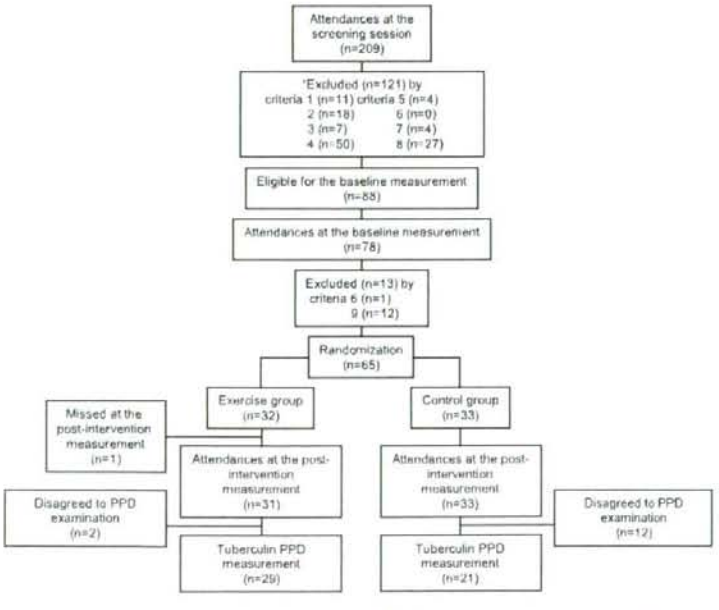

Fig. 1. Flow chart showing numbers, training interventions, and outcome measures of the randomized exercise and control groups. *Exclusion criteria: *1*) moderate to severe motor impairment or neurological deficits; *2*) a history of coronary heart disease within 6 mo of the study; *3*) patients with hypertension (systolic blood pressure >160 mmHg or diastolic blood pressure >100 mmHg); *4*) joint pain or arthritis limiting the full range of motion at shoulder, elbow, hip, or knee joints; *5*) mental or other conditions that could interfere with participation; *6*) other chronic disease that could interfere with participation; *7*) history of fracture of a lower extremity or injurious falls within 6 mo of the study; or *8*) use of antihypertensive agents, antiarrhythmic agents, nitroglycerides, digitalis, or vitamin supplements. PPD, purified protein derivative.

Table 1. *Baseline characteristics of the subjects*

	C Group (n = 33)	ET Group (n = 32)	P Value
Age, yr	66.9±3.0	67.3±4.8	0.71
Female sex, %	54.6	53.1	0.91
Height, cm	157.8±9.2	157.0±7.4	0.73
Weight, kg	58.2±8.5	57.5±9.8	0.81
Vo_{2max}, ml·kg^{-1}·min^{-1}	24.7±4.9	23.7±5.7	0.71

Values are means ± SD. C, control; ET, exercise training; Vo_{2max}, maximal oxygen uptake. P values with Student's t-test

predicted age-adjusted maximum heart rate (220 − age). Heart rate was monitored throughout the endurance training to check whether exercise of the prescribed intensity was accomplished. The initial intensity of exercise was set at 5–20 W. The subject increased the exercise intensity under the supervision of exercise trainers to reach the target heart rate at fifth minute of exercise. When the heart rate exceeded the target by 5% heart rate reserve for more than 2 min, exercise intensity was reduced until subjects reached their target heart rate.

Resistance exercise session. Five types of exercise using rubber film manufactured for resistance exercise (Thera-Band Resistive Exerciser, Hygienic, Akron, OH) were performed in a five-stage incremental program under the supervision of an expert exercise trainer. Side raise for supraspinatus and deltoid muscles, elbow flexion for biceps muscle, knee extension for quadriceps muscle, hip abduction, and hip adduction were each repeated 20 times per session. Rubber films of various strengths were coded by colors and adjusted to appropriate lengths for each participant and each exercise. The strengths of the rubber films were selected so that each set of 20 repetitions exhausted the loaded muscle (20 repetitions maximum). In the last class of each stage, each participant was asked to repeat the exercise until exhaustion. If the participant could repeat it more than 25 times, we increased the strength of the rubber film for the next stage. The resistance training session was periodized to avoid chronic fatigue of the participants as well as the endurance training sessions. To minimize excessive fatigue, the subjects performed only three types of resistive exercise in the initial 5 wk followed by 5 wk of secondary acclimatization stage in which exercise intensity or the strength of rubber films was increased within a low range so that the participants could perform resistive training with medium level of effort without exhaustion or muscle soreness. Four-week building-up phases started from 11th wk. After 11th wk, at every 4 or 5 wk exercise frequency or rubber film strength was reduced to allow recovery from muscle soreness or fatigue (15th, 19th, and 22th wk).

Safety considerations. To ensure safety, a research nurse and a physician checked the health status and vital signs of each participant before and after each class, and they attended the classes (29).

Control group. We provided two 2-h classes a month to avoid dropouts. The participants were asked to attend classes at least once a month. The classes consisted of a 1-h lecture, the topic of which was not related to physical exercise, and 1 h of seated recreational activity. Otherwise, they were asked to continue their daily activities.

Vo_{2max} measurement. Vo_{2max} was estimated by stepwise incremental exercise on a bicycle ergometer. Participants were told to pedal at a constant rate of 60 ± 10 rpm. The initial workload was set at 10 W. The workload was increased thereafter 5–30 W stepwise each 2 min until the participant reached the age-adjusted target heart rate (85% age-adjusted maximal heart rate). Oxygen uptake every minute during the incremental exercise session was recorded using an expired-gas analyzer (AeroMonitor 280, Minato Medical Science, Osaka, Japan). Vo_{2max} was estimated by linearly extrapolating the oxygen uptake as a function of heart rate up to age-adjusted maximal heart rate.

Blood sampling. Blood samples were obtained before and after the training period and analyzed. Blood sampling was performed at least 1 wk after and no later than 2 wk after the last exercise bout. No participant had a medical history of allergy. Participants refrained from any moderate or heavy exercise for at least 12 h before blood sampling. No food or drink other than water was allowed for 10 h before blood sampling. Blood samples were drawn from antecubital veins in the morning between 8:00 and 10:00 AM, with sterile syringes containing 100 IU of heparin (Novo Nordisk, Copenhagen, Denmark) per 10 ml of blood for either mononuclear cell preparation or plasma separation, and with sterile syringes without anticoagulants for serum separation.

Total and differential white blood cell count. Total white blood cell (WBC) count was measured by using a Sysmex microcell counter SE-9000 (Sysmex, Kobe, Japan). The proportions of lymphocyte, monocyte, neutrophil, eosinophil, and basophil in peripheral blood samples were evaluated by the standard morphological technique using May-Giemsa-stained samples. The absolute number of each cell type was calculated from the total WBC count and the percentage of each type.

Lymphocyte subsets staining. Heparinized peripheral blood was stained with biotin-labeled anti-CD3, FITC-labeled anti-CD4 monoclonal antibody (mAb), and phycoerythrin-labeled anti-CD8 mAb. Isotype controls were used in this assay to account for the background binding of Ig. All the mAbs were purchased from BD Biosciences (San Jose, CA). After 30 min incubation with monoclonal antibodies at 4°C, cells were washed twice in ice-cold PBS, and then incubated with streptavidin-RED670 (Gibco BRL, Grand Island, NY) to visualize biotinylated antibody. After a 20 min incubation with streptavidin-RED670 at 4°C, heparinized peripheral blood was hemolyzed with FACS lysing solution (BD Biosciences). Cells were then washed twice in ice-cold PBS and were analyzed by three-color flow cytometry (BD Biosciences).

Serum concentration of IgE and IgG subclasses. Serum concentration of IgE and IgG subclasses was measured by fluoroenzyme immunoassay (Pharmacia and Upjohn, Uppsala, Sweden) or ELISA (Zymed Laboratories, San Francisco, CA).

Skin reaction to tuberculin PPD. A 0.1-ml saline solution containing 0.05 mg of tuberculin PPD (Japan BCG Laboratory, Tokyo, Japan) was injected intradermally at the anterior aspect of the forearm, avoiding superficial veins, by an experienced medical doctor using the standard clinical procedure. After the injection, participants were told not to undertake vigorous exercise until the skin reaction was evaluated. Skin reaction was evaluated 48 h after the injection by measuring the longitudinal and horizontal dimensions of both the palpable induration and the skin flush with a pair of vernier calipers by an experienced medical doctor who was blinded to the group assignments of the participants. The areas of both the skin indurations and the skin flush were calculated as the area of an oval. Skin reaction was also photographed together with a scale for future review. The same

Table 2. *Exercise prescription for endurance training*

Week No.	Phase	Target Intensity of Exercise, % HR reserve	Duration of Exercise, min
1	Educational 1	<25†	10
2–4	Educational 2	50	10
5–9	Buildup 1	50	15
10	Evaluation	††	††
11–12	Buildup 2a	50	20
13–15	Buildup 2b	60	20
16	Recovery 1	40	20
17–19	Buildup 3	60	20
20	Recovery 2	40	20
21–25	Buildup 4	60	25

HR, heart rate. †Workload was fixed for educational purpose. ††Subjects' HR response to exercise was evaluated by the same protocol as in the baseline measurement.

lot of PPD preparation was used throughout the study. Participants who exhibited hyperreaction with extended swelling and skin ulceration underwent clinical assessment for tuberculosis infection, including breast X-ray examination and examination of C-reactive protein. Hyperreactive participants were excluded from posttraining PPD examination. PPD skin test was performed at least 1 wk after and no later than 2 wk after the last exercise bout.

Statistical analysis. Values shown in the tables are presented as means ± SE. ANOVA for two (group) × two (time) repeated measures was used to determine the effect of treatment during the 25-wk period between each group. All post hoc comparisons were performed using Fisher's positively least significant difference test. A χ^2 test was performed to test the differences in the proportion of PPD nonresponders and responders between groups at baseline. All statistical analyses were performed with StatView software (SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

RESULTS

Throughout the 25 wk of intervention, there were no dropouts from either group. The mean attendance rate for each class was 78.2% in the ET group and 84.5% in the C group. In the ET group, 28 subjects (82%) attended at least twice in a week throughout the 6-mo program. In the C group, 31 subjects (94%) attended their classes at least once a month throughout the 6-mo program. One woman was unable to attend the postintervention measurement session for a nonmedical personal reason. Analyses were therefore based on 31 participants from the ET group and 33 C group participants (Fig. 1).

Physical characteristics of participants. Estimated $\dot{V}O_{2\,max}$ increased from 25.6 ± 5.3 to 27.1 ± 5.1 $ml^{-1} \cdot kg \cdot^{-1} min$ after 25 wk of exercise training (F value = 4.44, $P < 0.05$). There was no significant difference in estimated $\dot{V}O_{2\,max}$ before and after 25 wk in the C group. There was no significant difference in the other parameters before and after exercise training in either groups.

Cell count. Blood samples from 62 out of 64 participants (ET: men/women = 14/15; C: men/women = 15/18) were analyzed; three blood samples were excluded from the analysis because of apparent hemolysis and possible clotting. As shown in Table 3, the mean values of circulating cell counts at baseline were not significantly different between the ET and C groups. Within the ET group, counts of WBC (before: 5,009.4 ± 234.0 and after: 5,458.7 ± 273.7), and neutrophils (before: 2,545.6 ± 166.9, and after: 3,216.7 ± 185.8) were increased after 25 wk of exercise. Blood lymphocyte count (before: 2,064.6 ± 130.0 and after: 1,826.3 ± 112.4) was decreased after 25 wk of exercise, Within the C group, counts of WBC (before: 4,670.6 ± 146.4 and after: 5,067.6 ± 156.7) and neutrophils (before: 2,065.7 ± 104.0 and after: 2,963.3 ± 134.1) were increased after the 25-wk exercise period. Blood lymphocyte count (before: 2,204.9 ± 110.6 and after: 1,784.9 ± 102.8) was decreased after 25 wk of exercise, Between-group analysis showed that circulating cell counts were not significantly different before and after exercise training.

Table 4. *Changes in the percentage of CD4 and CD8 T-lymphocyte subsets*

	Group	Before, %	After, %	Interaction (Time × Group)	
				F Value	*P* Value
$CD4^+$ $CD8^+$	C	3.8±0.5	3.5±0.5	1.53	0.22
	ET	2.9±0.7	3.0±0.7		
$CD4^+$ $CD8^-$	C	55.3±2.3	51.9±1.6	0.35	0.56
	ET	53.6±2.0	51.5±2.2		
$CD4^-$ $CD8^+$	C	35.2±1.5	38.9±1.8	0.20	0.66
	ET	35.2±2.0	38.0±2.3		
$CD4^-$ $CD8^-$	C	6.5±0.9	6.6±0.7	0.49	0.49
	ET	8.2±1.2	7.5±1.3		

Values are means ± SE. Effect of intervention over 25-wk period between each group was analyzed by ANOVA for 2 (group) × 2 (time) repeated measures.

Circulating T-lymphocyte subsets. As shown in Table 4, the mean values of T-cell subsets at baseline were not significantly different between the ET and C groups. Within the ET group, the percentage of $CD4^+$ $CD8^-$ (53.6 ± 2.0 to 51.5 ± 2.2) was decreased, and $CD4^-$ $CD8^+$ (35.2 ± 2.0 to 38.0 ± 2.3) was increased, after the 25-wk exercise period. Between-group analysis showed that circulating T-cell subsets were not significantly different before and after exercise training.

Skin reaction of tuberculin PPD. Physical characteristics of the ET and C group participants who agreed to undergo PPD skin testing were not significantly different at baseline. Skin induration of 5 mm or more in diameter was considered to be a positive reaction. Four participants (2 from each of the ET and C groups) who developed a strong skin reaction accom-

Table 3. *Changes in the number of circulating leukocytes*

	Group	Before	After	Change (After − Before)		Interaction (Time × Group)	
				Volume	*P* Value	*F* Value	*P* Value
WBC, /μl	C	4,670.6±146.4	5,067.6±156.7	397.6±126.4	<0.01	0.08	0.78
	ET	5,009.4±234.0	5,458.7±273.7	449.4±137.4	<0.01		
Lymphocyte, /μl	C	2,204.9±110.6	1,784.9±102.8	−420.0±100.0	<0.01	1.81	0.18
	ET	2,064.6±130.0	1,826.3±112.4	−238.3±90.2	0.02		
Monocyte, /μl	C	233.4±22.3	162.9±14.2	−70.5±25.7	<0.01	1.41	0.24
	ET	253.1±26.3	231.1±28.1	22.0±32.0	0.53		
Neutrophil, /μl	C	2,065.7±104.0	2,963.3±134.1	897.7±137.8	<0.01	1.60	0.21
	ET	2,545.6±166.9	3,216.7±185.8	671.1±112.2	<0.01		
Eosinophil, /μl	C	131.8±18.9	124.1±23.9	7.7±21.9	0.73	2.01	0.16
	ET	117.5±17.5	159.0±27.9	41.5±27.2	0.12		
Basophil, /μl	C	34.5±7.8	32.4±6.6	−2.0±8.2	0.81	0.01	0.93
	ET	28.7±5.7	25.6±6.4	−3.0±7.3	0.50		

Values are means ± SE. WBC, white blood cells. Time effect of intervention was analyzed by Student's *t*-test. Effect of intervention over 25-wk period between each group was analyzed by ANOVA for 2 (group) × 2 (time) repeated measures.

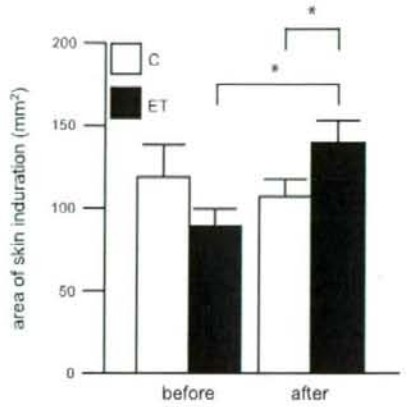

Fig. 2. Change in skin reactions to tuberculin PPD before and after the training period. Error bars represent SE of the mean. Twenty-nine participants from exercise training (ET) group and 21 participants from the control (C) group agreed to undergo tuberculin PPD examination. Among them, 4 participants (2 from each of the ET and C groups) who exhibited necrotizing skin inflammation did not undergo posttraining examination. Seven participants in the ET group and 4 participants in the C group were nonresponders to PPD, and they remained nonresponsive in the postintervention examination. PPD- delayed-type hypersensitivity responses of the participants excluding hyperresponsive and nonresponsive participants were analyzed (ET group: $n = 20$, men/women = 12/8; C group: $n = 15$, men/women = 8/7). The DTH response in the ET group after training was significantly higher than before training. However, there was no significant difference in DTH response before and after training in the C group. There was a significant group × time interaction in repeated-measures ANOVA analysis (*$P < 0.05$).

panied by necrotizing skin inflammation did not participate in the posttraining PPD test. Seven and four nonresponders in ET and C groups, respectively, demonstrated neither skin reaction nor induration of >5 mm in diameter. There were no statistical differences in the proportion of positive responders between the ET and C groups at baseline (χ^2 test) as well as at the posttraining assessment. Therefore, the analysis for the effect of exercise intervention involved 20 participants (men/women = 12/8) from the ET group and 15 participants (men/women = 8/7) from the C group, although there were no significant differences in major characteristics at baseline between these two groups.

After the training intervention, we found DTH response to tuberculin PPD was enhanced in the ET group but not in the C group (Fig. 2). There was a significant group × time interaction ($P < 0.05$) in repeated-measures ANOVA. In terms of skin responsiveness, both responders and nonresponders remained responders and nonresponders after the intervention. Their systemic inflammatory markers such as C-reactive proteins or WBC counts were within normal limits at the time of examination, and there were no signs of fresh or reactivating lung tuberculosis (data not shown).

Concentration of serum IgE and IgG subclasses. The mean values of serum IgE and IgG subclass concentrations at baseline were not significantly different between the ET and C groups. There were no significant differences in the serum concentration of IgE and IgG2 subclasses before and after training in either group (Table 5). Levels of IgG1 and IgG3 were decreased significantly after training, but there was no significant group differences. The level of serum IgG4 was decreased significantly in the ET group after training, whereas it was unchanged in the C group. The group difference in the change in serum IgG4 was statistically significant ($P < 0.05$).

DISCUSSION

The aim of this substudy of a randomized controlled trial for investigation of health benefits of exercise training on elderly participants was to examine whether health-oriented exercise training normally used to improve cardiovascular fitness would affect tuberculin PPD responses in healthy elderly people. Although not all the subjects underwent immunological assessment, enhanced DTH reaction of tuberculin PPD and reduced serum IgG4 concentrations were found among the exercise participants posttraining, which was not observed among the C group subjects, who did not attend exercise sessions.

Whereas tuberculin PPD skin reaction is specific antigen dependent, serum IgG4 may depend on wide spectrum of unknown antigens. Nevertheless, immunodeficient patients with mutant GATA-3, a well-established transcription factor crucial for Th2 differentiation (31) demonstrate markedly decreased serum IgG4 concentration as well as scarcely detected Th2 cytokine-producing cells in the bloodstream (25). Serum IgG4 level may therefore be considered as a reflection of Th2 immunity. Simultaneous enhancement in the skin reaction to tuberculin PPD and the reduction of serum IgG4 after 6 mo

Table 5. *Changes in serum IgE and IgG subclass concentration*

	Group	Before	After	Change (After − Before)		Interaction (Time × Group)	
				Volume	P Value	F Value	P Value
IgE, IU/ml	C	94.1 ± 25.7	131.2 ± 34.2	37.8 ± 23.7	0.06	0.26	0.61
	ET	94.2 ± 32.6	118.0 ± 41.6	23.8 ± 12.4	0.13		
IgG1, μg/ml	C	8,381.4 ± 372.8	7,191.4 ± 493.9	−1,190.1 ± 409.9	<0.01	0.65	0.42
	ET	8,316.7 ± 605.5	7,652.0 ± 495.1	−664.7 ± 511.2	0.02		
IgG2, μg/ml	C	4,111.2 ± 261.6	4,079.0 ± 240.6	−32.2 ± 115.3	0.78	2.87	0.10
	ET	3,688.4 ± 315.7	4,051.1 ± 253.6	362.7 ± 207.0	0.09		
IgG3, μg/ml	C	372.3 ± 50.1	307.5 ± 40.6	−64.8 ± 23.7	0.01	1.16	0.06
	ET	494.9 ± 102.1	377.8 ± 67.9	−117.1 ± 43.1	0.01		
IgG4, μg/ml	C	459.9 ± 74.6	455.3 ± 73.9	−4.6 ± 20.0	0.82	4.38	0.04*
	ET	457.5 ± 97.8	345.4 ± 56.4	−112.0 ± 54.9	0.05		

Values are means ± SE. Time effect of intervention was analyzed by Student's t test. The effect of intervention over 25-week period between each group was analyzed by ANOVA for 2 (group) × 2 (time) repeated measures. *$P < 0.05$, ANOVA and Fisher's positively least significant difference test.

training in this study thus seems to be in accordance with the reciprocal regulation of Th1 and Th2 immune responses. We, therefore, suggest chronic exercise training in healthy elderly people appears to favor in vivo Th1 immune responses.

In this study, serum IgG4 was decreased after training and no change was observed in the C group. However, the levels of IgG1 and IgG3, both known to be Th1 dependent, significantly decreased after 25-wk training period both in the ET group and the C group. Because postintervention blood sampling was performed later than 1 wk after the last exercise session, we assume that the acute effect of exercise was negligible. Baseline measurement was in March, and postintervention measurement was in October. We assume this common decrease in IgG1 and IgG3 may be because of the seasonal variation, because the decrease was common to both groups accompanied by increases in the neutrophil count and decreases in the lymphocyte counts. Seasonal effect on Th1 and Th2 immune responses will be an interesting focus of investigation in scope of allergic diseases due to pollen and other plant components.

Anti-influenza IgG and IgM levels in response to vaccination were reported to be greater in the active population, who engaged in more than 20 min of vigorous exercise three or more times per week, compared with the moderately active population, who engaged in regular exercise with less intensity, frequency, or duration, or sedentary populations (15). Because the initial IgG and IgM responses to vaccines or viral infection are considered to be Th1 dependent (24), greater anti-Influenza IgG and IgM response in the active population may be associated with enhanced Th1 capacity as observed in this study.

Two cross-sectional studies have examined the relationships between physical activity or fitness and DTH response. Smith et al. (26) found that the physically active older group had significantly higher anti-keyhole limpet hemocyanin IgM, IgG, IgG1, and DTH responses, but not IgG2, compared with the sedentary older group. Keylock et al. (14) found no differences between fitness groups in DTH skin test responses but did find differences to two fungal antigens, trichophyton and candida (14). Although these studies had controls for differences in health, psychosocial status, and nutrition, cross-sectional studies by their nature could be limited.

Another study examined the effect of exercise and food intervention on DTH responses in frail elderly people. That study reported a significant decline in DTH responsiveness in nonexercising subjects compared with unchanged responsiveness in exercising subjects (5). They did not find the enhancement in DTH response that we did in our exercising subjects. One of the reasons that they could not demonstrate improvement may be that their subjects were on average more than 10 yr older than our subjects. However, considering the age-related decline in immune response, both sets of results suggest that exercise training may prevent or slow the aging process.

The first phase of the DTH reaction involves uptake, processing, and presentation of the antigen by local antigen-presenting cells. Dendritic cells (DC) are likely to play a key role in immunity to *Mycobacterium tuberculosis*. DC exhibit the unique ability to ingest pathogens at the site of infection and to migrate to secondary lymphoid organs, such as lymph nodes, where they present pathogen-derived antigens to naive T lymphocytes (1). Priming and activation of mycobacterial antigen-specific T lymphocytes are essential for protection against tuberculosis (9, 13). Acute exercise or surgical stress raises circulating DC counts (12). We could not assess an in vivo DC function or distribution for ethical reasons, and this is one of the limitations of this study.

Nakayama et al. (21) showed that elderly nonresponders to tuberculin PPD had a higher probability of developing pneumonia when compared with the responders (21). Our nonresponders to PPD remained nonresponders even after exercise training in this study. Therefore, we cannot simply conclude that enhanced DTH response in this study favors resistance to pneumonia. Although PPD reactivity is strongly associated with clinical outcome, the clinical significance of the enhanced DTH response remains to be determined.

There are two limitations in this study. First, we could not recruit all the study participants of Sendai Silver Center Trial into PPD measurement, especially in the C group. One of the reasons for the refusal may be because of the negative attitude towards tuberculosis related testing of the elderly people in Japan. In 1957, when the majority of the study participants were in their adolescence and young adults, the prevalence of registered tuberculosis patients for the age group of 20 in Japan was 409.9/100,000 (18). Nevertheless, smaller number of control participants could be a potential bias in the interpretation of our result. But, because we found no statistical difference in the distribution of PPD nonresponders, nor the following parameters that may potentially affect the exercise responses; $Vo_{2\,max}$, age, sex, body weight, and height, at baseline we considered our result still significant as a controlled study. Second, psychological stress modulates immunity response in the elderly. Higher levels of anger, fatigue, and overall mood disturbance are related to lower Th1 cytokine responses to live virus, whereas vigor and optimism are related to greater Th1 cytokine response to live virus (6). Because we could not collect information of psychosocial factors, psychological study is necessary to further understand the regulation of tuberculin PPD responses.

In conclusion, we demonstrated that a 25-wk program of moderate exercise training for the healthy elderly significantly enhanced tuberculin PPD skin DTH reaction, which depends on Th1 activation. PPD skin reaction was significantly enhanced only in the ET group, but exercise did not render nonresponders to PPD to responders. These data confirm and extend the existing literature regarding the influence of exercise training on immune responses and support the need for further cross-sectional studies of cell-mediated immune responses in older adults.

ACKNOWLEDGMENTS

This study was conducted by the Sendai Silver Center Trial study group (29).

GRANTS

This study was supported by research grants from Ministry of Education and Culture (09557034) and Ministry of Health and Welfare (Research on Health Services: H10-025), Japan.

REFERENCES

1. **Banchereau J, Steinman RM.** Dendritic cells and the control of immunity. *Nature* 392: 245–252, 1998.
2. **Bonifacio E, Scirpoli M, Kredel K, Fuchtenbusch M, Ziegler AG.** Early autoantibody responses in prediabetes are IgG1 dominated and suggest antigen-specific regulation. *J Immunol* 163: 525–532, 1999.
3. **Boucher N, Dufeu-Duchesne T, Vicaut E, Farge D, Effros RB, Schachter F.** CD28 expression in T cell aging and human longevity. *Exp Gerontol* 33: 267–282, 1998.

4. **Cher DJ, Mosmann TR.** Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. *J Immunol* 138: 3688–3694, 1987.
5. **Chin APMJ, de Jong N, Pallast EG, Kloek GC, Schouten EG, Kok FJ.** Immunity in frail elderly: a randomized controlled trial of exercise and enriched foods. *Med Sci Sports Exerc* 32: 2005–2011, 2000.
6. **Costanzo ES, Lutgendorf SK, Kohut ML, Nisly N, Rozeboom K, Spooner S, Benda J, McElhaney JE.** Mood and cytokine response to influenza virus in older adults. *J Gerontol A Biol Sci Med Sci* 59: 1328–1333, 2004.
7. **Ernst DN, Weigle O, Hobbs MV.** Aging and lymphokine gene expression by T cell subsets. *Nutr Rev* 53: S18–S25; discussion S25–S26, 1995.
8. **Fiorentino DF, Bond MW, Mosmann TR.** Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 170: 2081–2095, 1989.
9. **Flynn JL, Chan J.** Immunology of tuberculosis. *Annu Rev Immunol* 19: 93–129, 2001.
10. **Fong TA, Mosmann TR.** The role of IFN-gamma in delayed-type hypersensitivity mediated by Th1 clones. *J Immunol* 143: 2887–2893, 1989.
11. **Fujita K, Nagatomi R, Hozawa A, Ohkubo T, Sato K, Anzai Y, Sauvaget C, Watanabe Y, Tamagawa A, Tsuji I.** Effects of exercise training on physical activity in older people: a randomized controlled trial. *J Epidemiol* 13: 120–126, 2003.
12. **Ho CS, Lopez JA, Vuckovic S, Pyke CM, Hockey RL, Hart DN.** Surgical and physical stress increases circulating blood dendritic cell counts independently of monocyte counts. *Blood* 98: 140–145, 2001.
13. **Kaufmann SH.** How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* 1: 20–30, 2001.
14. **Keylock KT, Lowder T, Leifheit KA, Cook M, Mariani RA, Ross K, Kim K, Chapman-Novakofski K, McAuley E, Woods JA.** Higher antibody, but not cell-mediated, responses to vaccination in high physically fit elderly. *J Appl Physiol* 102: 1090–1098, 2007.
15. **Kohut ML, Cooper MM, Nickolaus MS, Russell DR, Cunnick JE.** Exercise and psychosocial factors modulate immunity to influenza vaccine in elderly individuals. *J Gerontol A Biol Sci Med Sci* 57: M557–M562, 2002.
16. **Lancaster GI, Halson SL, Khan Q, Drysdale P, Wallace F, Jeukendrup AE, Drayson MT, Gleeson M.** Effects of acute exhaustive exercise and chronic exercise training on type 1 and type 2 T lymphocytes. *Exerc Immunol Rev* 10: 91–106, 2004.
17. **Lundgren M, Persson U, Larsson P, Magnusson C, Smith CI, Hammarstrom L, Severinson E.** Interleukin 4 induces synthesis of IgE and IgG4 in human B cells. *Eur J Immunol* 19: 1311–1315, 1989.
18. **Ministry of Health, Labor and Welfare.** Comprehensive evaluation of tuberculosis. Ministry of Health, Labor and Welfare Database [Online], 2004, Tokyo, Japan. http://www.mhlw.go.jp/wp/seisaku/jigyou/04sougou/1-5-1html.
19. **Mosmann T, Cherwinski HR, Bond MW, Giedlin MA, Coffman RL.** Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136: 2348–2357, 1986.
20. **Mosmann TR, Sad S.** The expanding universe of T-cell subsets: Th1, Th2 and more [see comments]. *Immunol Today* 17: 138–146, 1996.
21. **Nakayama K, Monma M, Fukushima T, Ohrui T, Sasaki H.** Tuberculin responses and risk of pneumonia in immobile elderly patients. *Thorax* 55: 867–869, 2000.
22. **Ng WY, Thai AC, Lui KF, Yeo PP, Cheah JS.** Systemic levels of cytokines and GAD-specific autoantibodies isotypes in Chinese IDDM patients. *Diabetes Res Clin Pract* 43: 127–135, 1999.
23. **Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, de Waal Malefyt R, de Vries JE.** Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 90: 3730–3734, 1993.
24. **Rentenaar RJ, Gamadia LE, van DerHoek N, van Diepen FN, Boom R, Weel JF, Wertheim-van Dillen PM, van Lier RA, ten Berge IJ.** Development of virus-specific CD4(+) T cells during primary cytomegalovirus infection. *J Clin Invest* 105: 541–548, 2000.
25. **Skapenko A, Leipe J, Niesner U, Devriendt K, Beetz R, Radbruch A, Kalden JR, Lipsky PE, Schulze-Koops H.** GATA-3 in human T cell helper type 2 development. *J Exp Med* 199: 423–428, 2004.
26. **Smith TP, Kennedy SL, Fleshner M.** Influence of age and physical activity on the primary in vivo antibody and T cell-mediated responses in men. *J Appl Physiol* 97: 491–498, 2004.
27. **Sousa AO, Henry S, Maroja FM, Lee FK, Brum I, Singh M, Lagrange PH, Aucouturier P.** IgG subclass distribution of antibody responses to protein and polysaccharide mycobacterial antigens in leprosy and tuberculosis patients. *Clin Exp Immunol* 111: 48–55, 1998.
28. **Steensberg A, Toft AD, Bruunsgaard H, Sandmand M, Halkjaer-Kristensen J, Pedersen BK.** Strenuous exercise decreases the percentage of type 1 T cells in the circulation. *J Appl Physiol* 91: 1708–1712, 2001.
29. **Tsuji I, Tamagawa A, Nagatomi R, Irie N, Ohkubo T, Saito M, Fujita K, Ogawa K, Sauvaget C, Anzai Y, Hozawa A, Watanabe Y, Sato A, Ohmori H, Hisamichi S.** Randomized controlled trial of exercise training for older people (Sendai Silver Center Trial: SSCT): study design and primary outcome. *J Epidemiol* 10: 55–64, 2000.
30. **Widhe M, Ekerfelt C, Forsberg P, Bergstrom S, Ernerudh J.** IgG subclasses in Lyme borreliosis: a study of specific IgG subclass distribution in an interferon-gamma-predominated disease. *Scand J Immunol* 47: 575–581, 1998.
31. **Zheng W, Flavell RA.** The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587–596, 1997.

Brain, Behavior, and Immunity 23 (2009) 108–115

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Acute stress-induced colonic tissue HSP70 expression requires commensal bacterial components and intrinsic glucocorticoid

Kaori Matsuo [a], Xiumin Zhang [b], Yusuke Ono [c], Ryoichi Nagatomi [a,*]

[a] Department of Medicine and Science in Sports and Exercise, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Japan
[b] School of Public Health, Jilin University, China
[c] Randall Division of Cell and Molecular Biophysics, King's College London, London, UK

ARTICLE INFO

Article history:
Received 1 May 2008
Received in revised form 22 July 2008
Accepted 29 July 2008
Available online 11 September 2008

Keywords:
HSP70
ZO-1
Acute restraint stress
Commensal bacteria
TLR4

ABSTRACT

Induction of heat shock protein (HSPs) has a protective effect in cells under stress. Physical stressors, such as restraint, induce HSPs in colonic tissue *in vivo*, but the mechanism of HSP induction is not yet clear. Because commensal bacteria support basal expression of colon epithelial HSP70, we postulated that stress responses may enhance the interaction of commensal bacteria and the colonic tissue. Restraining C57BL/6 mice for 2 h effectively induced HSP70 in colonic epithelia. Both blockade of stress-induced glucocorticoid by RU486 or elimination of commensal bacteria by antibiotics independently abrogated restraint-induced HSP70 augmentation. Oral administration of LPS to commensal-depleted mice restored restraint-induced HSP70 augmentation. Because TLR4 expression was absent from the epithelial surface, and was limited to lamina propria and muscularis externa, we examined how LPS reaches the lamina propria. Alexa-LPS administered in the colonic lumen was only detected in the lamina propria of the restrained mice. Expression of the tight junction component ZO-1 in the epithelia, which regulates the passage of luminal substances through the epithelia, was reduced after restraint, but reversed by RU486.

In conclusion, HSP70 induction in colonic epithelial cells under restraint requires both stress-induced glucocorticoid and luminal commensal bacteria, and LPS plays a significant role. Glucocorticoid-dependent attenuation of epithelial tight junction integrity may facilitate the access of LPS into the lamina propria, where TLR4, known to be required for HSP70 induction, is abundantly expressed. Sophisticated regulation of colonic protection against stressors involving the general stress response and the luminal environment has been demonstrated.

1. Introduction

Stress episodes are important risk factors for the development and reactivation of intestinal inflammation in rodents (Qiu et al., 1999) and in humans (Levenstein et al., 2000; Stam et al., 1997). Both environmental and psychological stress are known to alter the clinical course of inflammatory bowel disease (IBD) (Danese et al., 2004; Kugathasan et al., 2007; Mawdsley and Rampton, 2005; Tanaka et al., 2007; Tlaskalova-Hogenova et al., 2004). General stress reactions, such as activation of the HPA axis upon acute stress, are commonly accepted as having protective effects (Ader et al., 1979; Besedovsky and Sorkin, 1977; Riley, 1981). However, it has not been well documented how such adaptational reactions occur or are disrupted in the colon.

One of the cellular protective measures against stressors is the induction of HSP expression. HSPs are highly conserved proteins found in all prokaryotes and eukaryotes (Tobian et al., 2004). HSPs serve as molecular chaperones and play a significant role in the protection of cells against various cellular stressors, such as heat (Cvoro et al., 1998; Evdonin et al., 2006; Tomasovic et al., 1983), hypoxia (Dwyer et al., 1989; Zimmerman et al., 1991), ultraviolet irradiation (Kwon et al., 2002; Trautinger et al., 1996), oxidative stress (Drummond and Steinhardt, 1987), or endoplasmic reticulum stress (Yoneda et al., 2004). Unless intracellular HSPs are induced to protect cells exposed to acute stressors, they may undergo apoptosis or necrosis (Yun et al., 1997).

HSPs are also induced in various tissues and organs in response to various psychophysiological stressors (Fleshner et al., 2004; Fukudo et al., 1997), such as restraint (Campisi et al., 2003), ischemia (Kukreja et al., 1994; Lee et al., 2006; Oksala et al., 2002), exercise (Fehrenbach et al., 2005), infection (Ramaglia et al., 2004), inflammation (Ludwig et al., 1999), and hyperthermia (Hotchkiss et al., 1993). Among the various HSPs, HSP70[1] has recently been shown

* Corresponding author. Fax: +81 22 7178589.
E-mail address: nagatomi@m.tains.tohoku.ac.jp (R. Nagatomi).

[1] *Abbreviations used:* HSP70, heat shock protein 70; ZO-1,zonula occludens-1; TLR4, Toll-like receptor 4; RU486, mifepristone; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay.

0889-1591/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbi.2008.07.013

to exhibit a protective role in gut epithelial cells (Liu et al., 2003). However, the mechanism by which such protective HSP70 is induced in the colon has not been determined.

We assumed roles of activation of the HPA axis and sympathetic nervous system in colon HSP70 expression as well as the contribution of commensal bacteria in stress-induced HSP70 expression, as basal HSP70 expression in gut epithelial cells has been reported to be dependent on commensal bacteria through interactions with TLR4 (Rakoff-Nahoum et al., 2004).

Interestingly, TLR4, which is responsive for bacterial lipopolysaccharides in the intestinal tissue, was shown to be located mainly at the lamina propria and absent at the epithelial surface (Rumio et al., 2006). TLR4 in the colonic tissue would also exhibit a similar distribution. For luminal bacterial components to interact with TLR4, we postulated that tight junction integrity of the gut epithelia may be altered under stress facilitating access of luminal bacterial components to the lamina propria TLR4 and finally inducing HSP70.

In the present study, therefore, we first examined colonic HSP70 under acute stress, and then examined the involvements of luminal bacteria and stress-induced corticosterone in HSP70 induction in colonic epithelia. In addition, we examined the expression of the tight junction component ZO-1 in the colonic tissue before and after stress.

2. Methods

2.1. Animals

Male C57BL/6 mice and green fluorescence protein (GFP) transgenic mice (C57BL/6 background) were purchased from the mouse supply centre of Tohoku University School of Medicine, and used for the experiments at 10–12 weeks of age. Five or six mice were housed together per cage (30 × 25 × 17.5 cm) and were given free access to food and water. Animals were maintained under specific pathogen-free conditions on a daily 12-h light/dark cycle. Drinking bottles, cages, sawdust, and rodent chow for mice were all autoclaved before use as described previously (Kanemi et al., 2005). Mice were housed in freshly sterilized cages with fresh sawdust every day. All experiments were performed in accordance with the Guidelines and Regulations for Laboratory Animal Care of Tohoku University Medical School.

2.2. Restraint stress procedure

The stressor used in this study was restraint (Fukui et al., 1997; Kanemi et al., 2005; Sudo et al., 1997). Each experimental male C57BL/6 or GFP mouse was placed for 2 h in 50-ml centrifuge tubes. The walls of the tubes were stripped in part to avoid an acute rise in the body temperature. The restraint experiment was carried out in the morning between 8:00 and 10:00. Non-restraint mice of the same age were kept in their home cage throughout the restraint procedure. Animals were given no access to food or water during restraint stress treatment. Immediately after restraint stress, restrained and non-restrained mice were sacrificed by anesthesia.

2.3. In vivo RU486 and propranolol treatment

The glucocorticoid receptor antagonist RU486 was administrated orally (30 mg/kg) in aqueous solution containing 0.25% carboxymethylcellulose and 0.2% polysorbate 80 (Sigma, St. Louis, MO, USA) in a volume of 5 ml/kg through a gastric feeding tube (Concordet and Ferry, 1993; Zhang et al., 2005a,b).

RU486 or an equivalent volume of vehicle (0.25% carboxymethylcellulose and 0.2% polysorbate 80) was given 30 min before the 2-h restraint session.

2.4. Depletion of colonic commensal bacteria

Depletion of colonic commensal bacteria was performed according to the method reported by Rakoff-Nahoum et al. (2004) with minor modifications by daily administration of 0.2 ml of the following antibiotic cocktail *via* a ball-tipped gastric feeding tube for 7 days and by supplementing the drinking water with a 2-fold dilution of antibiotic cocktail during the same 7-day period. The antibody cocktail contained ampicillin (1 mg/ml), vancomycin (0.5 mg/ml), neomycin sulfate (1 mg/ml), and metronidazole (1 mg/ml) (all from Sigma).

2.5. Lipopolysaccharide (LPS) administration to antibiotic-treated mice

Following antibiotic treatment, mice were given a daily supplement of 0.2 ml distilled water containing 50 μg/ml of purified *Escherichia coli* 026:B6 LPS (Sigma) through a gastric feeding tube for 5 days. Drinking water during this period was supplemented with LPS (25 μg/ml). A lower dose of LPS did not induce stable augmentation of colonic HSP70. Mice in the control group received the same volume of distilled water according to the same protocol.

2.6. Anesthesia

Sevoflurane (Abbott Japan, Tokyo, Japan) inhalation was used to anesthetize the animals. A volume of less than 2 ml of anesthetic was evaporated into a container of approximately 500 ml at room temperature. The mice were then placed into the container and anesthetized within 15 s (Kanemi et al., 2005).

2.7. Blood sampling, catecholamines, and corticosterone assay

Blood samples were collected from the axillary artery of anesthetized mice and transferred immediately into either heparinized or untreated stock tubes for plasma and serum sampling, respectively. After blood sampling, animals were euthanized by deep anesthesia with sevoflurane.

Blood samples were centrifuged at 3000 rpm for 10 min at 4 °C to obtain plasma or serum. Plasma and serum samples were stored at −80 °C until assay. Catecholamines were quantified by column-switching high-performance liquid chromatography (HPLC) with fluorometric detection (Dutton et al., 1999; Nohta et al., 1987). Serum corticosterone level was measured using a rat corticosterone-H^3 RIA kit (ICN Biomedicals, Costa Mesa, CA, USA), in accordance with the manufacturer's instructions.

2.8. Rectal temperature

The rectal temperature of the mice was measured with a digital thermometer (CTM-303 model; Terumo, Kanagawa, Japan) at a distance of 2.5 mm from the anus. Measurements were obtained at ambient room temperature (20–23 °C). The rectal temperatures of control and stressed mice were measured at 30-min intervals, for a total of 120 min ($n = 5$).

2.9. Bacterial culture

For determination of colonic microflora, fecal matter was removed from the colon using sterile technique. The contents were diluted and plated on universal and differential media for the growth of anaerobes and aerobes under anaerobic or aerobic culture conditions. AnaeroPack (Mitsubishi Gas Chemical Company, Niigata, Japan) was used to detect anaerobic flora. The numbers of colonies per mg of feces (wet weight) were counted after incubation at 37 °C for 48 h (aerobes) and 72 h (anaerobes).

2.10. Colonic tissue preparation

The mid-portion, approximately 1 cm, of total colon—transverse colon—was excised immediately after the restraint session and stored at −80 °C until examination. After determining the frozen weight of colon samples, colonic tissue was lysed with lysis buffer consisting of 40 mM Tris (pH 7.5), 300 mM KCl, 1% Triton X-100, 2 mM EDTA, with protease inhibitor cocktail (1:20) (Sigma). Colonic tissue was dissected using scissors and further homogenized using a Polytron Aggregate homogenizer (Kinematica, Luzern, Switzerland). The extracts were cleared by centrifugation at 21,000*g* for 10 min. Aliquots of prepared samples were kept frozen at −80 °C until ELISA or Western blotting analysis.

2.11. ELISA for HSP70

HSP70 concentration in the extract was quantified by enzyme-linked immunosorbent assay (ELISA) using a commercially available HSP70 kit (StressXpress HSP70 ELISA; Stressgen Biotechnologies, Victoria, BC, Canada) in accordance with the manufacturer's instructions.

2.12. Western blotting analysis

Equal amounts of protein from the supernatant of colonic tissue samples quantified by the Lowry method were electrophoresed on 12% SDS–polyacrylamide gels and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk in phosphate buffered saline (PBS) containing 0.1% Tween 20 for 1 h at room temperature, membranes were incubated with rabbit polyclonal anti-HSP70 antibody (1:1000) (Stressgen), rabbit polyclonal anti-TLR4 (H-80) antibody (1:500) (Santa Cruz Biotechnology, CA, USA), rat polyclonal anti-ZO-1 antibody (1:250) (Santa Cruz), and mouse monoclonal anti-β-actin antibody (1:2000) (Sigma) in TBS (pH 8.0) containing 0.05% Tween 20 (TBST) at 4 °C overnight and washed three times for 5 min with PBS. The membranes were then incubated with either HRP-conjugated secondary anti-rat IgG (1:1000) or anti-mouse IgG (1:1000) (Cell Signaling Technology, MA, USA) antibody in TBST for 1 h at room temperature, washed three times for 5 min with PBS, and visualized by chemiluminescence using an ECL immunoblotting kit (Cell Signaling) with a digital luminescent image analyzer (LAS-1000; Fujifilm, Tokyo, Japan).

2.13. Immunohistochemistry

The colon samples from male C57BL/6 mice were rapidly embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen in isopentane, pre-cooled in liquid nitrogen, and finally transferred into liquid nitrogen. The tissue blocks were stored at −80 °C prior to cutting. Colonic tissues on slides were fixed in PBS containing 4% paraformaldehyde at room temperature for 20 min, blocked, and penetrated in blocking solution (Dako, Kyoto, Japan) containing 0.3% Triton X-100 for 30 min at room temperature, and incubated overnight with rabbit polyclonal anti-HSP70 antibody (1:500, Stressgen), goat polyclonal anti-TLR4 (L-14) antibody (1:200) (Santa Cruz), and rat polyclonal anti-ZO-1 (R40.76) antibody (1:50) (Santa Cruz) in TBS (pH 8.0) containing 0.01% Triton X-100 at 4 °C overnight. Washes were performed with PBS. The sections were incubated with the secondary antibodies, Alexa Fluor 555-conjugated donkey anti-rabbit IgG (1:400) (Invitrogen, Carlsbad, CA), Alexa Fluor 488-conjugated donkey anti-goat IgG (1:400) (Invitrogen), and Alexa Fluor 488-conjugated goat anti-rat IgG (1:400) (Invitrogen) with nuclear stain marker Topro-3 in TBS (pH 8.0) containing 0.01% Triton X-100 for 1 h at room temperature. The reaction was examined by confocal microscopy (Nikon, Tokyo, Japan).

2.14. Ligated colon loop assays and processing of colonic tissues

Mouse ligated colon loop assays were performed as described previously by Mantis et al. with some modifications (Mantis et al., 2002). During the procedure, mice were maintained under sevoflurane anesthesia. Alexa-LPS 548 was injected into ligated colon loops (1 cm) at a concentration of 1/10 μl. After 10 min, mice were sacrificed by cervical dislocation and the colon was excised immediately. Portions of tested loops were taken and fixed in Tissue-Tek OCT compound (Sakura Finetechnical), frozen in isopentane, pre-cooled in liquid nitrogen, and finally transferred into liquid nitrogen. Colon sections of the specimens were prepared using standard procedures for immunohistochemical analyses.

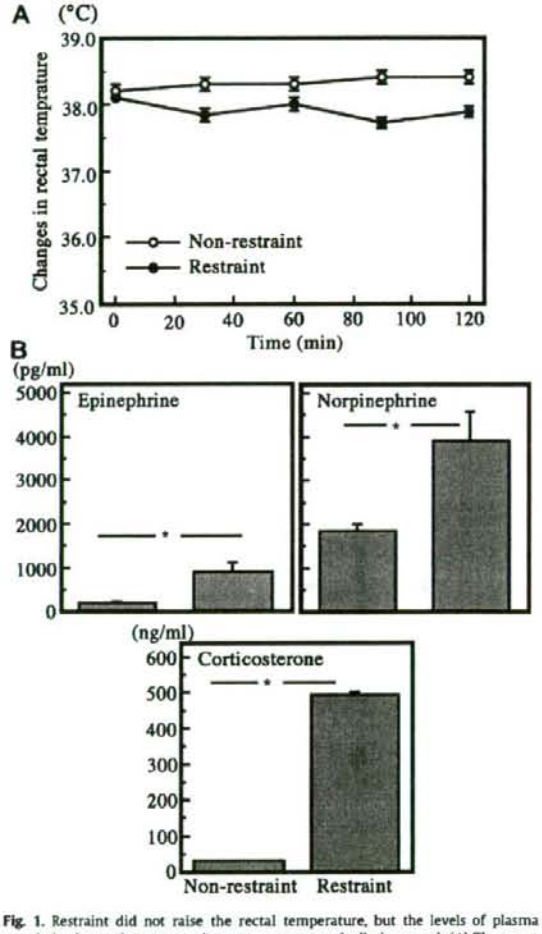

Fig. 1. Restraint did not raise the rectal temperature, but the levels of plasma catecholamine and serum corticosterone were markedly increased. (A) The mean rectal temperature of mice decreased during restraint stress. The results are shown as means ± SE of 5 mice ($^*P < 0.05$). (B) Plasma levels of epinephrine and norepinephrine was significantly elevated immediately after restraint stress. Results are shown as means ± SE of 6 mice ($^*P < 0.001$). Serum level of corticosterone was significantly elevated immediately after restraint stress. The results are shown as means ± SE of 5 mice ($^*P < 0.001$).

2.15. Statistical analysis

Results are presented as the means ± SE. We examined the statistical significance of differences using the two-way analysis of variance (ANOVA). *Post hoc* analysis was carried out using Scheffe's test. Statistical significance was defined as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Restraint stress did not raise the rectal temperature of mice

First, we examined whether there was a rise in rectal temperature in response to restraint stress, because a rise in the rectal temperature may involve HSP70 induction in the colonic tissue (Fig. 1A). The mean rectal temperature of mice decreased gradually during restraint stress, while there was virtually no change in the rectal temperature of non-restrained mice ($n = 5$, $P < 0.05$).

3.2. Restraint stress-induced elevation of plasma catecholamine and corticosterone levels

The plasma levels of epinephrine and norepinephrine were significantly elevated immediately after restraint stress ($P < 0.05$, Student's *t*-test) (Fig. 1B).

The serum level of corticosterone was significantly elevated immediately after restraint stress ($P < 0.001$, Student's *t*-test) (Fig. 1B).

The plasma levels of epinephrine and norepinephrine of antibiotics treated mice were significantly elevated immediately after restraint stress (non-restraint: epinephrine 260.0 ± 50.7 pg/ml, norepinephrine 1806.0 ± 593.9 pg/ml *vs.* restraint: epinephrine 1157.5 ± 193.2 pg/ml, norepinephrine 4438.8 ± 184.0 pg/ml, $n = 4$; $P < 0.05$, Student's *t*-test).

The serum level of corticosterone of antibiotics treated mice was significantly elevated immediately after restraint stress (non-restraint: 36.8 ± 12.7 ng/ml, restraint: 501.1 ± 28.5 ng/ml, $n = 4$; $P < 0.001$, Student's *t*-test).

Thus, antibiotics treatment did not affect the neurohumoral stress response to restraint.

3.3. Restraint stress enhances colonic HSP70

The effects of 2-h restraint on colonic HSP70 were examined both by ELISA (Fig. 2A) and by immunohistochemical analysis (Fig. 2B). HSP70 level in the colonic extract was significantly elevated immediately after restraint as compared to the non-restraint group ($P < 0.001$) (Fig. 2A). Immunohistochemical analyses indicated a detectable basal level of HSP70 expression, but that it was dominantly expressed at the apical or luminal cytoplasm of colonic epithelia. HSP70 expression was weaker in the cells in deeper crypts as compared to those closer to the tips (Fig. 2B). This expression pattern was unchanged after restraint stress, but the degree of HSP70 expression was enhanced after restraint.

3.4. Glucocorticoid receptor antagonist suppressed restraint-induced colonic HSP70 augmentation

Blockade of glucocorticoid by oral administration of RU486 before restraint completely abolished the augmentation of colonic HSP70 and even resulted in down-regulation to below the level of expression in non-restrained mice (basal level) (Fig. 2C). There were no significant differences in HSP70 level between non-restrained and restrained mice treated with RU486 (NS, Fig. 2C). Immunohistochemical analysis revealed no apparent morphological differences between RU486-treated and untreated colon tissue notwithstanding restraint (Fig. 2D). Colonic HSP70 augmentation after restraint was absent in RU486-treated mice.

3.5. Depletion of commensal bacteria abrogates restraint-induced colonic HSP70 augmentation

As commensal bacteria contribute to HSP expression in the gut tissue (Rakoff-Nahoum et al., 2004), the involvement of commensal bacteria in stress-induced HSP70 augmentation was examined.

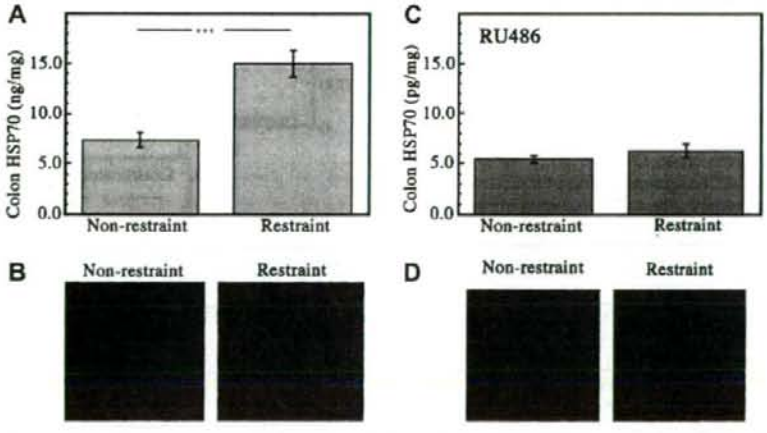

Fig. 2. Colonic HSP70 expression was markedly augmented after 2-h restraint. The augmentation of HSP70 expression was abrogated by RU486 administration. (A) The level of colonic HSP70 was significantly elevated immediately after restraint as compared with non-restrained controls. The results are shown as means ± SE of 8 mice ($^{***}P < 0.001$). (B) Immunohistochemical analysis indicated concentrated expression of colonic HSP70. HSP70: red; Topro-3: blue (200×). (C) No significant difference was found in the HSP70 level between non-restrained and restrained mice treated with RU486. The results are shown as means ± SE of 8 mice. (D) Immunohistochemical analysis revealed no apparent morphological differences in the levels of colonic HSP70 between non-restrained and restrained mice treated with RU486. No HSP70 augmentation after restraint was observed in RU486-treated mice. HSP70: red; Topro-3: blue (200×). There was a significant group × time interaction in repeated-measures ANOVA analysis ($p = 0.0005$, $F = 15.4$).