Volume Regulation by Human Lymphocytes

IDENTIFICATION OF DIFFERENCES BETWEEN THE TWO MAJOR LYMPHOCYTE SUBPOPULATIONS

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ABSTRACT Following exposure to hypotonic media, human peripheral blood lymphocytes swell initially but restore their isotonic volume within minutes. In contrast, tonsillar lymphocytes demonstrate a similar initial phase of swelling but fail to restore their isotonic volume. We have studied the ionic basis for this second or regulatory volume decrease (RVD) phase using lymphocytes from peripheral blood, tonsil, and thymus. RVD was characterized by ^{86}Rb efflux and a decrease in K^+ content. The increase in K^+ permeability in response to hypotonic challenge was characteristic for T lymphocytes obtained from peripheral blood, tonsil, or thymus. B lymphocytes showed only a modest increase in K^+ permeability and consequently little RVD. The data confirm that the response of peripheral blood and tonsillar lymphocytes to a hypotonic environment can be accounted for by differences in the proportions of T and B cells, and the differential behaviour of B and T lymphocytes is based on differences in membrane permeability to K^+ upon swelling.

INTRODUCTION

When subjected to hypotonic challenge, many cell types have the ability to restore their volume after an initial phase of passive swelling (1–9). This regulation of cell volume is mediated through shifts in cellular ion and water content (1, 3, 7). The shrinking phase or regulatory volume decrease (RVD)[1] occurs very rapidly after initial swelling in human lymphocytes (4, 8). Indeed, in contrast to erythrocytes (9), peripheral blood lymphocytes can readjust their volume very quickly, despite a much lower anion permeability, suggesting that different mechanisms may be operative in different cell types.

We have studied volume regulation by peripheral blood mononuclear cells (PBM) and confirmed the central role of potassium ions (K^+) in this process (8). During RVD, intracellular K^+ content decreased while Na^+ content remained essentially unchanged. The findings suggested that RVD occurs largely through the passive loss of cellular K^+, resulting from a selective increase in permeability to this ion.

PBM cell suspensions are heterogeneous and are comprised of distinct subpopulations of lymphocytes. Because the subsets of lymphocytes have been shown, in a variety of ways, to have unique membrane properties and functional activities (10–12), we studied the responses of circulating and tonsillar T and B lymphocytes and intrathymic T lymphocytes to hypotonic stress. We show that RVD is distinctly different in T and B cells, regardless of the tissue of origin. The ionic basis of these differences was analyzed by ion flux and content measurements.

METHODS

Cell preparations. Heparinized blood was obtained from healthy individuals, tonsil tissue from otherwise healthy children undergoing tonsillectomy, and thymus tissue from children at the time of cardiac surgery. Mononuclear cells were obtained following Ficoll-Hypaque gradient centrifugation (13). The isolated mononuclear cells were suspended in RPMI 1640 supplemented with 10% fetal calf serum at a concentration of 10^7 cells/ml. Peripheral blood mononuclear cell suspensions generally contained 10–20% monocytes, identified by nonspecific esterase staining (14). Monocyte depletion (<1% monocytes) was accomplished following adherent cell depletion on plastic petri dishes (15).

Separation of lymphocyte subpopulations was achieved by E-rosette depletion on Ficoll-Hypaque gradients as described (13). The E^+ (T) cell population contained >95% E-rosetting cells using PBM or tonsillar cells; the E^- (or B cell)

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[1] *Abbreviations used in this paper*: HTC, human tonsillar cells; PBM, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RVD, regulatory volume decrease.

preparations contained <1% E-rosetting cells. Tonsil E^- preparations contained >85% surface immunoglobulin staining cells and PBM E^- suspensions consisted of ~50% such cells unless monocytes were first depleted.

Cell viability, estimated both by trypan blue dye exclusion and ^{51}Cr release were identical for PBM and human tonsillar cells (HTC) at a given dilution of medium. At 0.7× isotonic medium, there was little effect on cell viability (>90% viability), and most of the experiments were performed with this diluted medium. If more dilute medium was used, correction for viability was made.

Cell volume determinations. Cell volume determinations were carried out using a Coulter counter model ZBI, with attached channelyzer (Coulter Electronics Inc., Hialeah, FL). Actual volumes were calculated from the median of volume distribution curves. Calibration was based on the use of latex beads of known diameter in solutions of appropriate salt concentration. Using the shape factor determined by Segel et al. (16), we estimated the average lymphocyte volume to be 205 μm^3 in isotonic solutions.

All experiments were carried out at room temperature.

Reagents. ^{86}Rb and ^{51}Cr were purchased from Amersham Corp. (Arlington Hts., IL) and ^3H_2O and [^{14}C]polyethylene glycol (mol wt 4,000) were from New England Nuclear, Boston, MA.

Media. Phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4, 8.1 mM $NaHPO_4$, 10 mM glucose, 0.68 mM $CaCl_2$, and 0.49 mM $MgCl_2$. K^+-rich medium was made by substituting the NaCl with KCl isoosmotically. The pH of the media was 7.3. Hypotonic media were prepared by diluting the isotonic media with the indicated amounts of deionized distilled water.

Determination of cell water content. Cellular water content was determined by isotope dilution using [^{14}C]-polyethylene glycol as an extracellular marker. 10^7 cells were suspended in a medium containing 10 μCi/ml of ^3H_2O and 1 μCi/ml [^{14}C]polyethylene glycol. Aliquots of this suspension were diluted at zero time with the indicated volume of PBS or hypotonic PBS. At appropriate time intervals, duplicate aliquots of the suspension were removed and layered over 0.4 ml of an oil mixture (corn oil/dibutyl phthalate, 3:10) and then centrifuged in an Eppendorf microfuge, to which a variable transformer was attached. Centrifugation was for 30 s at 50 V followed by 1 min at 100 V. Aliquots of the supernatant were saved for counting. The rest of the supernatant and most of the oil were discarded, and the tip of the tube containing the cell pellet covered by a thin layer of oil was cut off and transferred to a scintillation vial. The pellet was dissolved in 1 ml of 1% sodium dodecyl sulfate before addition of 10 ml of Aquasol II (New England Nuclear) and counting. Cell water space was determined by subtracting the calculated trapped extracellular space from the total pellet water content.

^{86}Rb efflux determinations. Cells were equilibrated overnight with 10 μCi/ml of ^{86}Rb in RPMI 1640 supplemented with 10% fetal calf serum. The cells were then diluted 10-fold in nonradioactive medium and pelleted at 350 *g* for 5 min. The cells were resuspended in PBS at ~10^7 cells/ml, and aliquots of this suspension were removed and processed as described above for cell water determinations.

Intracellular K^+ content. Cellular K^+ content was determined by flame photometry (photometer model 443, Instrumentation Laboratory, Inc., Lexington, MA) using Li^+ as an internal standard. The cells were suspended in media of the indicated osmolarity. At appropriate intervals, duplicate aliquots containing 4–6 × 10^6 cells were centrifuged as described above. The supernatant was removed and the side of the tube was rinsed three times with a standard solution of Li^+ (15 meq/liter $LiNO_3$). After removing the oil phase, the pellet was lysed in 1 ml of Li solution and the concentration of K^+ determined by comparison with standards. No correction was made for K^+ trapped in the extracellular space because the amount was negligible.

RESULTS

Response of PBM and HTC to hypotonic stress. When incubated in hypotonic medium, normal PBM (from >50 donors) demonstrated the ability to regulate their cell volume. In contrast, the majority of tonsillar lymphocytes (HTC) failed to do so. Fig. 1 illustrates the volume changes of PBM and HTC exposed to a hypotonic medium with a 30% reduction in osmolarity. Studies using both electronic sizing and cellular water content measurements resulted in essentially identical patterns for both tissues. However, whereas PBM regained near normal volume in ~15 min, little or no RVD was observed in HTC, even after 30 min. Removal of monocytes by adherence depletion, or elimination of platelets by defibrination of peripheral blood (as opposed to the use of heparin), did not affect the results. Similarly, addition of heparin to HTC suspensions had no effect on RVD.

The patterns of response of PBM and HTC were maintained through the whole range of diluted media tested (Figs. 2A, B). As expected, 1 min after dilution, before the regulatory mechanism becomes apparent, the increase in relative volume was essentially the same for PBM and HTC, with the increment dictated by the degree of dilution of the medium.

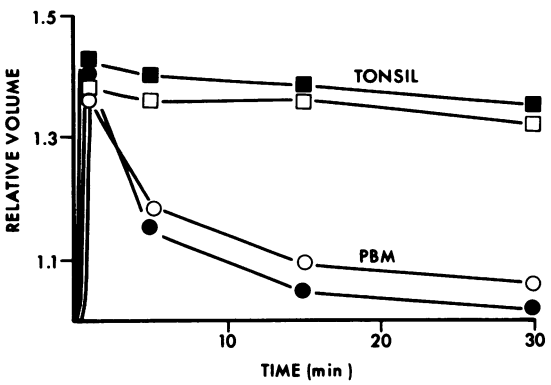

FIGURE 1 Relative changes in cell volume determined by electronic sizing (closed symbols), and cell water content (open symbols), during RVD. The volume and water content of cells in isotonic medium are taken as unity. At zero time, PBM or tonsillar cells were subjected to hypotonic stress (0.7× isotonic). Data represent the means of three experiments, each carried out in duplicate.

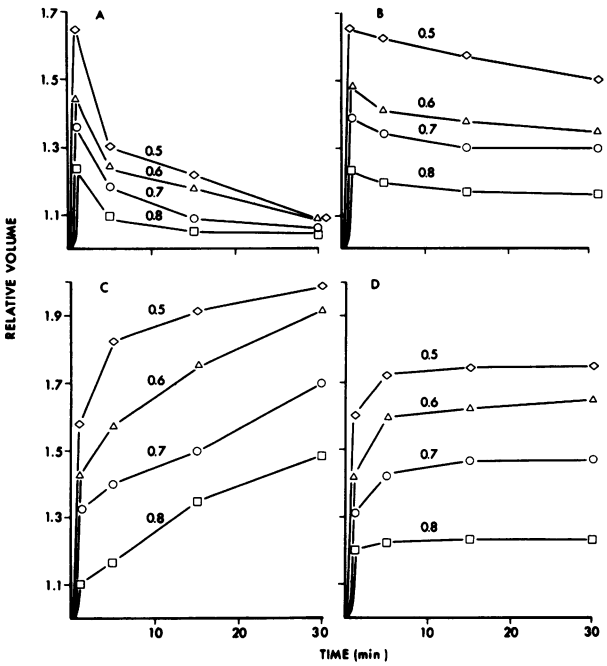

FIGURE 2 Relative changes in median cell volume during RVD in media of varying dilutions. PBM (A) and HTC (B) were exposed to sodium-containing media of the indicated relative osmolarity or to media with K^+ substituted for Na^+ [PBM (C), HTC (D)]. The values for each curve indicate the concentration of the medium relative to isotonic medium. The cell volume in isotonic PBS is taken as unity. Data represent the means of five experiments, each carried out in duplicate.

Isotonic replacement of external Na^+ by K^+ did not alter cell volume during the experimental period (30 min). In hypotonic K^+-rich media where the electrochemical K^+ gradient is essentially abolished, RVD was not seen. As shown (8), the rapid initial swelling of PBM (Fig. 2C) was followed by a slower secondary swelling. The degree of secondary swelling of HTC (Fig. 2D) was substantially less than that observed with PBM, suggesting a lower permeability of these cells to the cation.

Intracellular K^+ content and ^{86}Rb efflux. Loss of K^+ during RVD is a major response of PBM after hypotonic challenge (8). With increasing dilution of the medium, the K^+ content of PBM progressively decreased (Fig. 3A). In contrast, with HTC (Fig. 3B) the decrease in K^+ content was significantly lower and not progressive after 10–15 min.

^{86}Rb was used as a K^+ analogue for unidirectional flux measurements, because it is also transported by the same pathways as K^+ in volume-static (17) and volume-regulating cells (8). ^{86}Rb efflux from cells in isotonic media was slow, with <10% of the isotope lost over the 30-min observation period (Fig. 4). In both PBM and HTC, ^{86}Rb efflux in hypotonic media resembled the reduction in K^+ content. In 0.7× isotonic medium, roughly 25% of the isotope was lost by PBM, whereas only 10% was lost from HTC, and the time course of ^{86}Rb efflux paralleled the loss of K^+ and RVD.

Response of lymphocyte subpopulations to hypotonic stress. The contrasts observed between PBM and HTC could be explained by differences in the

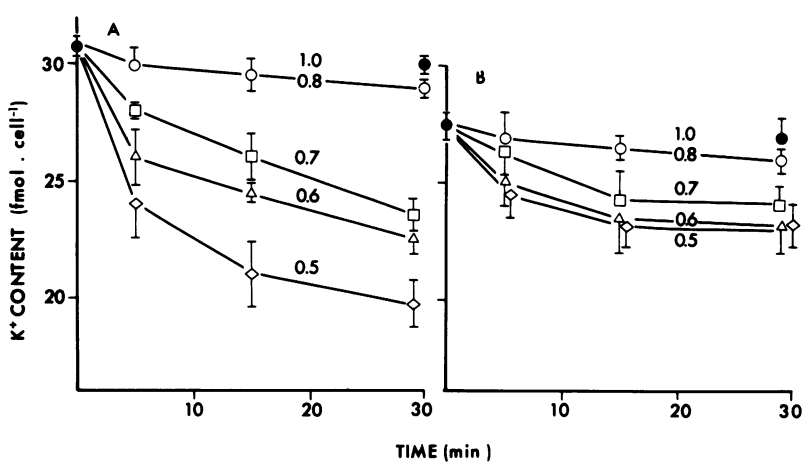

FIGURE 3 Changes on cellular K^+ content in response to different degrees of hypotonic stress. PBM (A) and HTC (B) were suspended in media of the indicated relative osmolarity. Samples were taken at the indicated time interval and K^+ content was measured. The closed symbols represent the values in isotonic media (×1.0 isotonic). The results (mean±SE) of four separate experiments are shown.

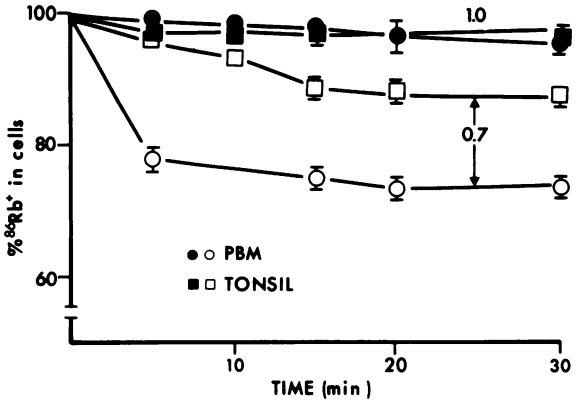

FIGURE 4 Effect of hypotonic stress on ^{86}Rb efflux. The cells were loaded overnight with ^{86}Rb, followed by removal of extracellular isotope by centrifugation. The loaded cells were suspended in either isotonic (1.0× isotonic) or hypotonic (0.7× isotonic) medium and centrifuged as described in Methods at the indicated time intervals. The ordinate indicates percent ^{86}Rb remaining in cells. Data represent mean±SE of six experiments.

composition of the cell suspensions; PBM contain ~70% T cells and 10% B lymphocytes (and 20% monocytes); HTC contain roughly 20–25% T cells and 60% B cells. To further investigate this possibility, populations of T cells and B cells were obtained by rosette-depletion and gradient centrifugation. Electronic sizing (Fig. 5) and cell water determinations (data not shown) showed that T cells from peripheral blood (Fig. 5A) or tonsil (Fig. 5C) were capable of restoring their volume after initial swelling. The response of the former was complete at all dilutions by 15 min, whereas T cells from tonsil had a slower RVD, never quite reaching isotonic volumes. B cells from peripheral blood (Fig. 5B) or tonsil (Fig. 5D) failed to demonstrate significant RVD. Overall the responses of peripheral blood T cells and tonsillar B cells resulted in patterns virtually identical to those of PBM and HTC, respectively.

^{86}Rb efflux and K^+ content in lymphocyte subpopulations. The results of ^{86}Rb efflux studies (Fig. 6) and K^+ content (Table I) in purified B or T cells closely paralleled cell volume determinations. The results of ^{86}Rb determinations with tonsillar (Fig. 6A) and peripheral blood (Fig. 6B) T cells were virtually indistinguishable; the E^- or B cell populations of both tissues lost <10% of the isotope over 30 min. Similarly, studies of K^+ content were entirely consistent with the results observed with the ^{86}Rb efflux assays (Table I).

Effect of hypotonic stress on human thymocytes. We next attempted to determine if the ability of T cells to regulate their volume in hypotonic media was a universal phenomenon for both mature and immature cells. Human intrathymic lymphocytes or thymocytes were used as the source of a relatively homogeneous population of immature T lymphocytes (10), and their volume was measured by either electronic sizing or water space determinations. When exposed to hypotonic media, thymocytes responded with an initial swelling followed by an RVD phase (Fig. 7). In less hypotonic media, RVD was similar to that observed for T cells in blood or tonsil. With increasing dilution however, the rate of RVD was slower and at ×0.5 isotonic, no RVD was observed. Some of the differences may be attributable to the fragility of the

TABLE I
K^+ Content in T and B Cells

Cell source		fmole cell^{-1} Time, *min* 0	5	30
Peripheral blood	E^+	30.0±0.5	26.2±0.6	23.9±0.6
	E^-	29.7±0.3	29.0±0.5	28.0±0.8
Tonsil	E^+	27.8±0.5	23.4±0.5	22.0±0.6
	E^-	26.2±0.5	25.0±1.0	24.5±0.7

K^+ content of separated $T(E^+)$ and $B(E^-)$ cells from peripheral blood and tonsil were carried out as described in Methods. The medium was ×0.7 isotonic. Results represent mean±SE of four experiments.

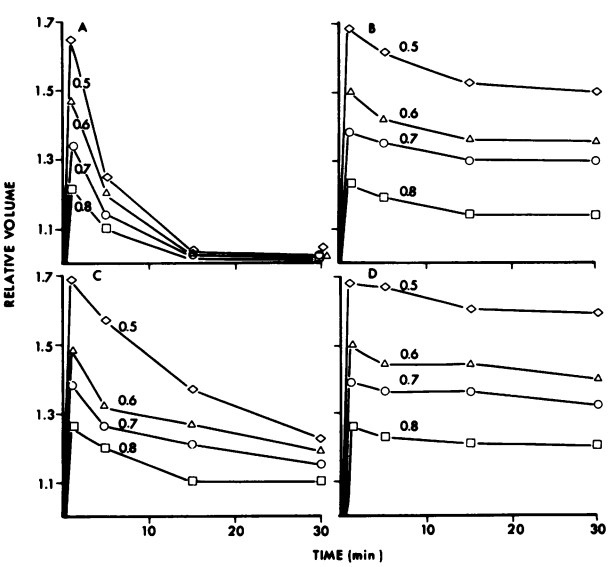

FIGURE 5 Relative changes in cell volume in hypotonic media for PBM E^+ (A) and E^- (B) cells or tonsil E^+ (C) or E^- (D). The volume of cells in isotonic medium is taken as unity. Cells were exposed to media of the indicated relative osmolarity. Data represent the means of three experiments for each group in duplicate determinations.

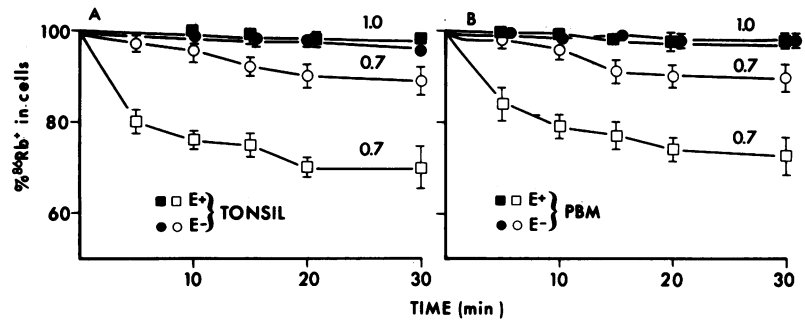

FIGURE 6 Effect of hypotonic stress on ^{86}Rb efflux for E^+ and E^- cells from tonsil (A) and PBM (B). The cells were loaded overnight with ^{86}Rb, followed by removal of extracellular isotope by centrifugation. The loaded cells were suspended in either isotonic (1.0× isotonic) or hypotonic (0.7× isotonic) medium and centrifuged as described in Methods at indicated time intervals. The ordinate indicates percent ^{86}Rb remaining in cells. Data represent the mean±SE of six experiments.

thymocytes, because considerable (35%) loss of viability occurred in the more diluted media.

The changes in cell volume of thymocytes were also paralleled by changes in ^{86}Rb content. During RVD there was progressive loss of ^{86}Rb (or K^+) (Fig. 8). When compared with T cells from blood or tonsil, the rate of ion loss was slower and did not attain the levels of the more mature T cells.

DISCUSSION

T and B lymphocytes have been shown to differ by many criteria. Both express a distinct array of surface receptors and ultrastructural features that are characteristic, at least in mature cells, of their specific lineage. More recently, these two populations have been characterized by differences in their capping response to lectins and colchicine (11). These data indicate that substantial differences exist in the membrane of the two major classes of lymphocytes. Importantly, both passive permeability and active transport of K^+ were found to be reduced in B cells as compared with T lymphocytes in isotonic media (18).

Although the entire molecular basis of the volume-induced regulatory pathway is not known, K^+ ions were found to play a central role in RVD after hypotonic stress (5, 8, 19). Thus, it was of interest to determine whether volume regulation is also different

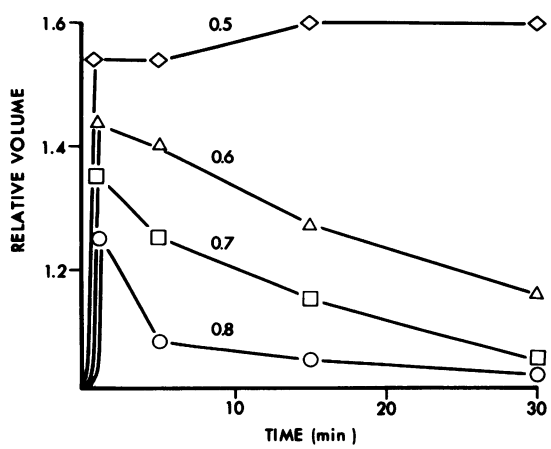

FIGURE 7 Relative changes in cell volume following incubation of human thymocytes in hypotonic medium. Cell volume was measured with the Coulter counter and channelyzer and cell water was calculated from the distribution of ^3H_2O using [^{14}C]polyethylene glycol as an extracellular marker. Data represent the means of three experiments carried out in duplicate.

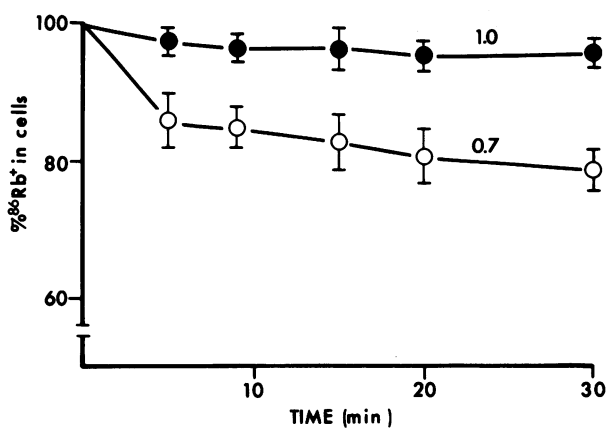

FIGURE 8 Effect of hypotonic stress on ^{86}Rb efflux from thymocytes. The cells were loaded overnight with ^{86}Rb, followed by removal of extracellular isotope by centrifugation. The loaded cells were suspended in either isotonic or hypotonic (0.7× isotonic) medium and centrifuged as described in Methods at indicated time intervals. The ordinate indicates percent ^{86}Rb remaining in cells. Data represent mean±SE of four experiments.

between T and B cells. The majority of studies on lymphocyte volume regulation have used peripheral blood mononuclear cells (4, 5, 8) or cell lines (1, 20). In one study, studies of chronic lymphocytic leukemia cells, which were of B cell origin, were shown to have a slower RVD phase, presumably due to decreased membrane permeability to K^+ (4, 18). These studies underlined the differences in the abilities of T and B cells to respond to osmotic challenge.

Our studies confirm that peripheral blood lymphocytes responded to hypotonic challenge by RVD by means of an increased K^+ permeability, detected as ^{86}Rb efflux. In contrast, tonsillar lymphocytes showed only a modest increase in K^+ permeability and consequently little RVD. Although peripheral blood and tonsillar cell suspensions contain both T and B lymphocytes, the peripheral blood cells are comprised predominantly of T cells, and tonsillar tissue of B lymphocytes, likely accounting for the particular responses of these tissues to hypotonic media. The failure to detect two populations of cells by electronic sizing, especially with HTC containing 20% of T cells, may (*a*) indicate the level of sensitivity of the assay and (*b*) reflect the fact that only median cell volumes were recorded. Some HTC suspensions (10–15%) did show relatively normal RVD responses. These preparations contained an abnormally high proportion of T cells. Overall the degree of RVD in HTC was directly correlated ($r = 0.94$) with the number of T cells in the particular preparation.

The information gained from the studies of peripheral blood and tonsils were confirmed when purified T and B cell preparations were studied directly. Using cell water determinations and electronic sizing, T cells from both blood and tonsil responded by initial swelling, followed by RVD. The kinetics of the RVD response for both T cell populations differed somewhat and at present are unexplained. As in the unfractionated HTC samples, B cells failed to respond with RVD or alterations in ion content. These findings are in contrast to those of Ben-Sasson et al. (4) who inferred, but did not directly test, the osmotic behavior of normal B lymphocytes.

We also determined whether immature T cells differed from mature cells when subjected to hypotonic stress. Human thymocytes serve as a relatively homogeneous population of T lymphocytes. The majority of these cells are "immature" when compared with peripheral blood or tonsillar T cells in assays of function, receptors, or expression of specific enzyme activities (10). However, their response to osmotic challenge was not easily distinguishable from their mature counterparts. The swelling phase at low dilutions was similar and, other than a slightly slower RVD phase, significant differences were not apparent between thymocytes and peripheral blood or tonsillar T cells. At high dilutions, the fragility of thymocytes became more obvious and affected cell viability.

Although these studies demonstrate that K^+ flow is an essential factor in RVD, our preliminary studies indicate that an increase in anion conductance is also required for the response to occur in peripheral blood cells. Further, anion conductivity, which is low in cells in isotonic media, is significantly enhanced upon swelling. Thus, it is conceivable that the deficient RVD in B cells is due to a lowered responsiveness of the volume-sensitive anion pathway. This does not appear to be the case: recent studies with gramicidin, a cation channel-former, indicate that the secondary swelling response of gramicidin-treated cells in hypotonic media is indistinguishable in T and B cells. This would imply that anion conductivities are similar in the two cell types and that a differential K^+ permeability is likely responsible for the differences in RVD responses.

However, the precise mechanism(s) underlying the gating of K^+ permeability pathways is not known. Previous work has suggested that cytoplasmic Ca^{++} is involved and that calmodulin may act as a regulator (21). The reduced responsiveness of B cells may then be explained by (*a*) a reduced number or turnover of K^+ transport sites, (*b*) a deficient release of Ca^{++} from intracellular storage sites upon swelling, or (*c*) differences in amounts or coupling of calmodulin to the K^+ transport system. Studies assessing these possibilities are in progress.

In summary, these results confirm the important role of K^+ in volume regulation. The data directly demonstrate that behavioral differences between peripheral blood and tonsillar lymphocytes can be accounted for by differences in the proportions of T and B cells. The differential behavior of B and T cells is based on differences in membrane permeability to potassium upon swelling. Thus the absence of RVD in B cells reflected the limited extent of their changes in potassium permeability. The response to hypotonic challenge may be acquired early in T-cell ontogeny because thymocytes clearly displayed RVD. Although the biological significance of volume regulation in lymphocytes remains to be defined, these experiments indicate that this particular property may be applied to studies directed at defining subpopulations of normal lymphocytes and differences between specific classes of leukemic lymphoblasts.

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