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PURINE METABOLISM AND B-LYMPHOCYTE DEVELOPMENT IN THE CHICKEN BURSA OF FABRICIUS

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□Abstract—The activity of three enzymes involved in the salvage pathway of purine nucleosides—purine nucleoside phosphorylase (PNP), xanthine dehydrogenase (XDH), and hypoxanthine-guanine phosphoribosyl transferase (HGPRT)—was investigated in cellular fractions of the chicken bursa of Fabricius differentially enriched in epithelial cells or lymphocytes. Markedly increasing levels of PNP and XDH were observed along with the enrichment in epithelial cells together with a slight, though significant, decrease in HGPRT activity. By contrast, a dramatic fall in PNP and XDH activities was detected along with the enrichment in lymphocytes together with a slight, though significant, increase in HGPRT activity. This sharply different distribution of the three enzymes, all sharing hypoxanthine as a substrate, clearly indicates that lymphocytes preferentially channel hypoxanthine into the salvage and interconversion pathways, phosphorylating it to IMP, while epithelial cells rapidly catabolize such a purine base to uric acid. Moreover, epithelial cells, unlike lymphocytes, are able to retain high intracellular levels of both hypoxanthine and inosine. These results support the possibility that epithelial cells contribute to the normal development of bursal lymphocytes by supplying such actively proliferating cells with purine rings and at the same time by preventing them from accumulating potentially toxic high levels of purine nucleotides being able to rapidly eliminate excess hypoxanthine as uric acid from the bursa environment into the bloodstream.

□Keywords—Purine enzymes; Purine metabolism and B-cell maturation; B-cell development; Chicken bursa of Fabricius.

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Introduction

Congenital deficiency of purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1; PNP), an enzyme involved in the purine salvage pathway, has been causally associated with severe immunological disorders in children (1–5), thus providing direct evidence for a key role of purine metabolic enzymes in the control of lymphocyte function. PNP deficiency mainly compromises the maturation of T lymphocytes, and extensive studies in various mammalian species have proved that different levels of the enzyme activity are related to distinct stages of T-cell differentiation and that PNP activity generally increases as T-cell maturation proceeds (6–8).

By contrast, the contribution of PNP to the development of B lymphocytes is still unclear (9–13). According to some reports, the B-lymphocyte hyporeactivity seen in PNP deficiency is most likely due to a lack of helper signals by PNP-deficient T lymphocytes rather than to a primary, PNP-dependent, impairment of the B lymphocyte itself (11).

A suitable model for investigating the role of PNP in the functional maturation of B lymphocytes is the bursa of Fabricius in birds since it represents a unique lymphoid organ where prebursal stem cells accumulate to give rise to a diversified population of mature B lymphocytes (14–17). The mechanism by which the bursa actually effects B-cell differentiation from precursor cells is largely speculative, although the epithelial cells are thought to be critically important as an inductive stimulus (18–20).

The PNP metabolic role is to catalyze the reversible phosphorolysis of purine nucleosides, inosine, xanthosine, and guanosine and of their 2′-deoxy-derivatives to the respective purine bases. Although PNP is an almost ubiquitous enzyme, the mechanism whereby its deficiency selectively affects lymphocyte function remains a matter of speculation. However, the abnormally increased levels of mono-, di-, and triphosphates, especially dGTP, which may rapidly accumulate within lymphocytes (10,12), are believed to be largely responsible for the immune hyporeactivity observed, at least in mammals (10–12).

The aim of this study was to evaluate the role of purine metabolism in the lymphocyte maturation process in the chicken bursa of Fabricius. For this purpose, the distribution of PNP in different bursal cell suspensions enriched in lymphocytes or in epithelial cells was studied together with that of other two enzymes that utilize the purine bases generated by the PNP enzymatic reaction in alternative metabolic fate: (i) xanthine dehydrogenase (xanthine: NAD^+ oxidoreductase, EC 1.1.1.204; XDH), which converts hypoxanthine and xanthine to uric acid, the only product responsible for the complete elimination of nitrogen in uricotelic animals, such as birds; and (ii) hypoxanthine-guanine phosphoribosyl transferase (IMP: pyrophosphate phosphoribosyl transferase, EC 2.4.2.8; HGPRT), which channels hypoxanthine and guanine into the salvage and interconversion pathways, phosphorylating them to IMP and GMP, respectively.

The results clearly show that in the chicken bursa the hypoxanthine produced by PNP undergoes a different metabolic fate in lymphocytes and epithelial cells, being preferentially recycled to IMP in the former and converted to inosine in the latter. The epithelial cells are also able to rapidly eliminate excess hypoxanthine as uric acid. Lymphocytes, unlike epithelial cells, are unable to retain high intracellular levels of both inosine and hypoxanthine. Therefore, epithelial cells appear to be critically important for the normal development of intrabursal lymphocytes, being able to supply such rapidly growing cells with purine rings, but also to prevent them from overproducing potentially toxic purine nucleotides by draining excess hypoxanthine from the bursa microenvironment.

Materials and Methods

Animals

Fertile eggs of the Arbor Acre chicken strain were obtained from a local hatchery and incubated as previously described (21). After hatching, chicks received food and water ad libitum and 3- to 4-week-old chicks were used in all the experiments.

Fractionation of Bursal Cells

Bursae were excised aseptically from four to five chicks and washed with PBS. Plicae were minced very gently with scissors and teased out through a steel strainer at 4°C in PBS. The resulting suspension, in which small fragments of tissue were still present, was washed twice at 500 × *g* for 10 min and referred to as

"whole bursa cell population" (WBCP). The suspension was allowed to sediment by gravity for 15 min at 4°C, thus giving rise to a supernatant fraction consisting of a single-cell suspension, mainly lymphocytes, referred to as "lymphocyte fraction I" (LFI), and a sediment fraction consisting of small particles of tissue, mainly showing an intact follicular medulla organization, referred to as "follicular fraction I" (FFI). The LFI was collected and the cells counted: Cell viability was usually higher than 97%, as assessed by nigrosin dye exclusion, and characterization of the cell morphology on hematoxylin/eosin stained slides showed 83 ± 4% lymphocytes and 17 ± 4% epithelial cells. The percentage of epithelial cells in this and in the subsequent bursal cell fractions was confirmed by staining some slides with the immunoperoxidase technique after treatment with an anticytokeratin mAb (Dakopatts, Glostrup, DK) (21). The FFI was washed once more with PBS and then hand homogenized in the same buffer in a loose-pestle homogenizer (Potter type) to free the cells from the follicular structures recovered in the fraction: The tissue fragments were gently pressed with the pestle (20–30 strokes) until a supernatant fraction was attained consisting of single cells released into the medium from the bursal follicles, the remaining of which readily sedimented out of the resulting suspension. The supernatant cells, referred to as "lymphocyte fraction II" (LFII), were collected while the sediment fraction, referred to as "follicular fraction II" (FFII), was washed twice by gravity sedimentation and the supernatants pooled. Cell viability of the LFII was 88–92% and the morphological characterization of the cells gave 87 ± 5% lymphocytes and 13 ± 5% epithelial cells.

The LFI was further enriched in lymphocytes by filtration through a glass-wool column, as described by Julius et al. (22). The effluent cells, referred to as "lymphocyte fraction III" (LFIII), were centrifuged and resuspended in PBS. The average cell recovery was 63 ± 7% and cell viability was 95–97%. More than 99% of the cells recovered in the LFIII were lymphocytes, as judged by their morphological features.

Neither the FFI nor the FFII were assessed for cell number and viability, owing to the particulate nature of such suspensions; however, morphological characterization of their cellular components was usually carried out following the procedure described by Boyd et al. (20); briefly, the tissue aggregates recovered in the FFI and FFII were digested to obtain a single-cell suspension by two to three cycles of incubation at 37°C for 20 min in a digestion mixture made up of RPMI 1640 medium, collagenase type IV (0.15%), trypsin (0.15%), and DNase (0.01%) (Boehringer Mannheim, GmbH, FRG); cell characterization gave 71 ± 6% lymphocytes and 29 ± 6% epithelial cells for the FFI, while 48 ± 7% lymphocytes and 52 ± 7% epithelial cells were counted in the FFII. The WBCP was treated in the same way as the FFI and FFII to obtain single-cell suspensions and 81 ± 6% lymphocytes and 19 ± 6% epithelial cells were counted.

Preparation of Whole Cell Homogenates

The bursal cell suspensions recovered in LFI, LFII, and LFIII were diluted to a final concentration of 2.0×10^8 cells/mL in cold PBS and homogenized by sonication with a BP-10 Blackstone Ultrasonic (Sheffield, PA). Sonication for 60–90 s in cycles of 30 s was usually effective in disrupting more than 99% of the cells, as seen at the light microscope. The bursal fractions containing tissue aggregates (WBCP, FFI, and FFII) were diluted in PBS (10 volumes of the buffer to 1 of the sedimented particles) and sonicated as described above. It was pre-

ferred to sonicate the tissue fragments rather than their cellular suspensions because of the high content of hydrolytic enzymes in the mixture used for the tissue digestion. All the homogenates were centrifuged at 15000 × *g* for 30 min at 4°C and supernatants were assayed for enzyme activities and protein content. The preparations were kept at 4°C and never stored below 0°C since a loss of PNP activity, up to 40–50%, occurred after only one cycle of freeze-thawing.

Enzyme Assays

All enzyme activities were assayed at 37°C by monitoring the formation of the reaction products in a Perkin-Elmer Lambda 5 spectrophotometer in 1.0-cm light-path semimicrocuvettes. Enzyme activities are expressed as nmoles of product formed/min per 10^8 cells in the LFI, LFII, and LFIII and per mg protein.

PNP activity was assayed according to Senesi et al. (23). The reaction mixture contained 0.2 mM inosine, 150 mM phosphate buffer, pH 7.2, excess xanthine oxidase from cow milk (Sigma Chemical Co., St. Louis, MO), and various amounts of enzyme preparation in a final volume of 1.0 mL. The formation of uric acid from inosine was followed at 293 nm, assuming an extinction coefficient of 11.26 $cm^{-1} \times mM^{-1}$ between inosine and uric acid.

XDH activity was measured as described by Strittmatter (24). The reaction mixture contained 0.27 mM xanthine, 0.67 mM NAD (Sigma), and 30 mM phosphate buffer, pH 7.5. The formation of uric acid was followed at 293 nm, assuming an extinction coefficient of 9.32 $cm^{-1} \times mM^{-1}$ between xanthine and uric acid.

HGPRT activity was assayed as described by Giovannitti et al. (25). The reaction mixture contained 0.5 mM 5-phosphoribosyl pyrophosphate, 0.11 mM guanine, 10 mM $MgCl_2$, and Tris-HCl 100 mM, pH 7.2, as a buffer. The assay was carried out at 257 nm by continuous spectrophotometric monitoring of the change in absorbance due to the conversion of guanine to GMP, assuming an extinction coefficient of 4.82 $cm^{-1} \times mM^{-1}$.

Protein Determination

Protein content was determined according to the method of Lowry et al. (26) using BSA as a standard. Standard curves were performed in PBS.

Statistical Analysis

Data are expressed as the mean ± SD of at least five separate experiments. One-way analysis of variance and the least significant difference test were used to evaluate the results statistically.

Results

Fractionation of Bursal Cells

Preliminary experiments were designed to develop a simple fractionation procedure for achieving bursa cell suspensions enriched either in epithelial cells or in lymphocytes. Such a procedure takes into account the architectural structure typical of the bursal follicles, which are made up of two main compartments, the cortex on the outside and the medulla inside, divided by a basement membrane and an epithelial layer (19,21), which are quite difficult to disrupt. By teasing out the bursa, essentially the cortical cells, mostly lymphocytes, which are held very loosely by the connective tissue, could be easily released into the medium (19), while the medullary lymphocytes remained enclosed inside the intact epithelial layer. These two fractions could be separately

collected simply by gravity sedimentation, as already described by Ratech et al. (27) and Senesi et al. (28). As confirmed at the light microscope, the LFI consisted of a single-cell suspension of lymphocytes (83%), most likely cortical lymphocytes, and few epithelial cells (17%) belonging to the covering epithelium; the FFI, on the other hand, was made up of bursal follicular medulla, the intact structure of which was maintained by the internal framework of reticular epithelial cells and the basement membrane. The high percentage of lymphocytes (71%) in the FFI should be essentially due to medullary lymphocytes. When the FFI was hand homogenized and gravity sedimented, a further enrichment in medullary lymphocytes (87%) was attained in the LFII, while the highest percentage of epithelial cells (52%) was recovered in the FFII where, however, a relatively high percentage of lymphocytes (48%) was still present. Further enrichment in epithelial cells from the FFII was not performed.

An almost pure lymphocyte suspension (99%) was obtained in the LFIII by glass-wool filtration of the LFI.

PNP, XDH, and HGPRT Content in the Different Bursal Cell Fractions

The levels of PNP in the various bursal cell fractions (Fig. 1) clearly showed that marked differences in PNP activity were associated with the fraction enrichment in lymphocytes or epithelial cells. As a matter of fact, the most striking difference was observed between the cell fractions enriched in epithelial cells, FFI and FFII, and the ones enriched in lymphocytes, LFI, LFII, and LFIII. The difference between the first two and LFIII was highly significant ($p < 0.001$). The difference was all the more evident between the FFII, the richest in epithelial cells, and the LFIII, the richest in lymphocytes, the activity in the latter being up to six times lower than in the former (4.92 ± 0.93 vs. 29.51 ± 4.23 nmol/min × mg protein). No significant difference was found between the PNP content of the LFI and LFII. As expected, PNP activity in the WBCP was an intermediate value between the FFII and the LFIII (12.65 ± 1.44 nmol/min × mg protein). Lymphocyte enrichment of the LFI led to a significant difference in enzyme content between the initial fraction and the effluent LFIII (7.82 ± 1.81 vs. 4.92 ± 0.93 nmol/min × mg protein; $p < 0.05$). A comparison of results expressed as nmoles of product formed/min per 10^8 cells was also performed for the populations where cell counts were possible, i.e., LFI, LFII, and LFIII: The difference in PNP content between LFI (15.62 ± 3.62 nmol/min × 10^8 cells) and LFIII (9.13 ± 1.81 nmol/min × 10^8 cells) was statistically significant ($p < 0.001$), while the difference between the former and the LFII (15.94 ± 3.42 nmol/min × 10^8 cells) was not. These data confirm those previously obtained comparing enzyme contents expressed as nmoles of product formed/min per mg protein for the same cell populations.

When XDH levels were compared, the distribution of this enzyme activity in the various cell fractions was found to vary very similarly to PNP since increasing amounts of XDH were detectable along with the enrichment in epithelial cells (Fig. 2). Indeed, similarly to PNP, the maximum amount of XDH was found in the FFII (5.20 ± 0.64 nmol/min × mg protein) while the lowest amount of enzyme was detected in the LFIII (0.16 ± 0.12 nmol/min × mg protein). Again, the WBCP was an intermediate value (1.45 ± 0.33 nmol/min × mg protein) between the two former fractions. As for PNP activity, there was a significant difference in XDH activity between the LFI and the FFII (0.52 ± 0.20 vs. 5.20 ± 0.64 nmol/min × mg protein; $p < 0.001$), between the LFII and the FFII (0.69 ± 0.29 vs. 5.20 ± 0.64 nmol/min × mg protein; $p < 0.001$), between the LFIII and the FFII

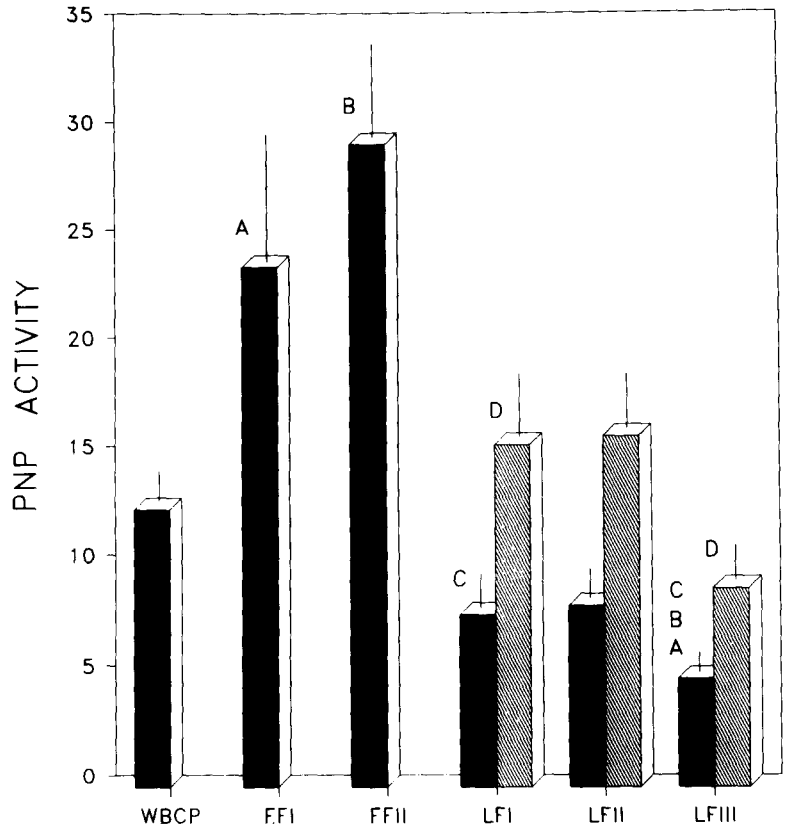

Figure 1. PNP activity in the WBCP and in bursal cell fractions enriched either in epithelial cells (FFI, FFII) or in lymphocytes (LFI, LFII, LFIII). PNP-specific activity is expressed as nmoles of product formed/min per mg protein (■) or per 10^8 cells (□). $p < 0.001$ between A, B, and C, respectively; $p < 0.05$ between D.

(0.16 ± 0.12 vs. 5.20 ± 0.64 nmol/min × mg protein; $p < 0.001$), and between the LFI and the LFIII (0.52 ± 0.20 vs. 0.16 ± 0.12 nmol/min × mg protein; $p < 0.05$); no significant difference in XDH activity was found between the LFI and the LFII (0.52 ± 0.20 vs. 0.69 ± 0.29 nmol/min × mg protein). Again, a similar behavior was observed when a comparison was made between XDH content in LFI, LFII, and LFIII given as nmoles of product formed/min per 10^8 cells: The difference in enzyme activity between the LFI and the LFIII (0.94 ± 0.47 vs. 0.30 ± 0.25 nmol/min × 10^8 cells) was statistically significant ($p < 0.05$), while the difference between the former and the LFII (1.19 ± 0.48 nmol/min × 10^8 cells) was not.

HGPRT showed no striking differences among the various cell fractions tested. However, statistically significant different levels of enzyme activity were observed between the FFII and the LFII (9.55 ± 0.94 vs. 14.0 ± 2.8 nmol/min × mg protein; $p < 0.01$) and between the FFII and the LFIII (9.55 ± 0.94 vs. 12.7 ± 0.9 nmol/min × mg protein; $p < 0.05$). No significant differences were found among the other cell fractions regardless of whether the enzyme activity was expressed per mg protein or per 10^8 cells (Fig. 3).

Discussion

The experimental procedure developed to fractionate the whole bursa cell population made it possible to obtain cel-

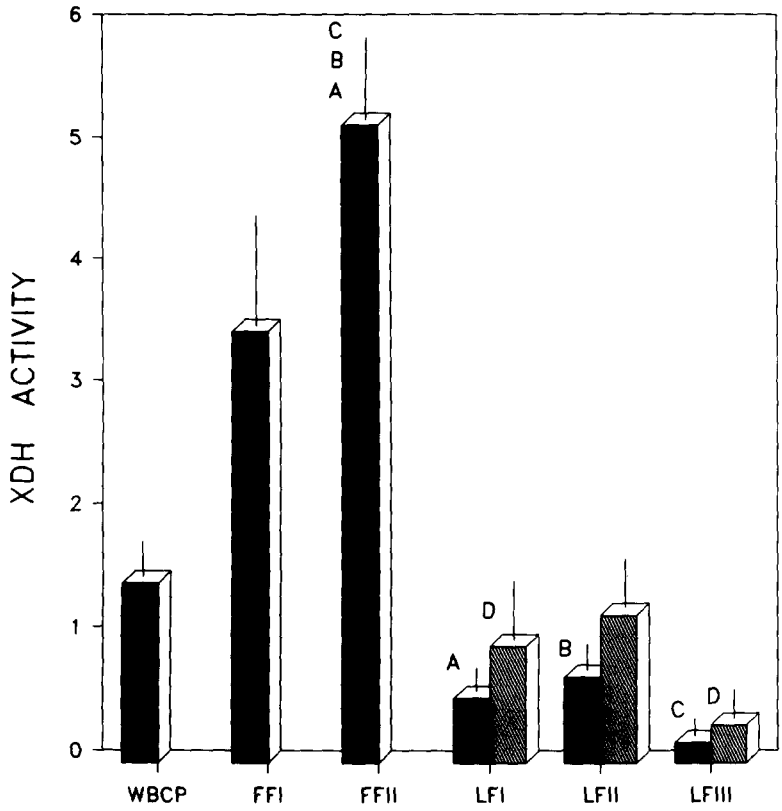

Figure 2. XDH activity in the same bursal cell fractions described in Fig. 1. XDH-specific activity is expressed as nmoles of product formed/min per mg protein (■) or per 10^8 cells (□). $p < 0.001$ between A, B, and C, respectively; $p < 0.05$ between D.

lular fractions differentially enriched in epithelial cells, the FFI and the FFII, or in lymphocytes, the LFI, the LFII, and the LFIII, the FFII and the LFIII resulting the richest in epithelial cells or lymphocytes, respectively. However, while the LFIII consisted of an almost pure lymphocyte population, the maximum enrichment in epithelial cells achieved in the FFII was never higher than 52%. Nevertheless, the procedure described enabled us to detect the changes in PNP, XDH, and HGPRT activity along with the enrichment in either type of cells.

The significantly increasing levels of PNP and XDH detected in the FFI and FFII, the fractions successively enriched in epithelial cells, clearly show that such cells do contain higher amounts of both enzyme activities than do lymphocytes. As a confirmation, a consistent decrease in PNP and XDH activities was found in all the lymphocyte-enriched fractions, with the lowest level observed in the LFIII, the cellular fraction virtually void of epithelial cells, in which especially the XDH activity was hardly detectable. These findings strongly suggest that in the chicken bursa the production of uric acid from purine rings is accomplished by epithelial cells rather than by lymphocytes (Fig. 4). As a matter of fact, the amount of XDH detected in the FFII is consistent with a high rate of production of uric acid in the epithelial compartment of the bursa from where it flows into the bloodstream. In this context, it may be of interest the finding that the amount of XDH in the liver and kidney of chicken, the primary organs involved in the elimination of nitrogen as uric acid in such animals, is twice as high as in the FFII (29,30), where, however, roughly 50% of the cells were lymphocytes, which lack

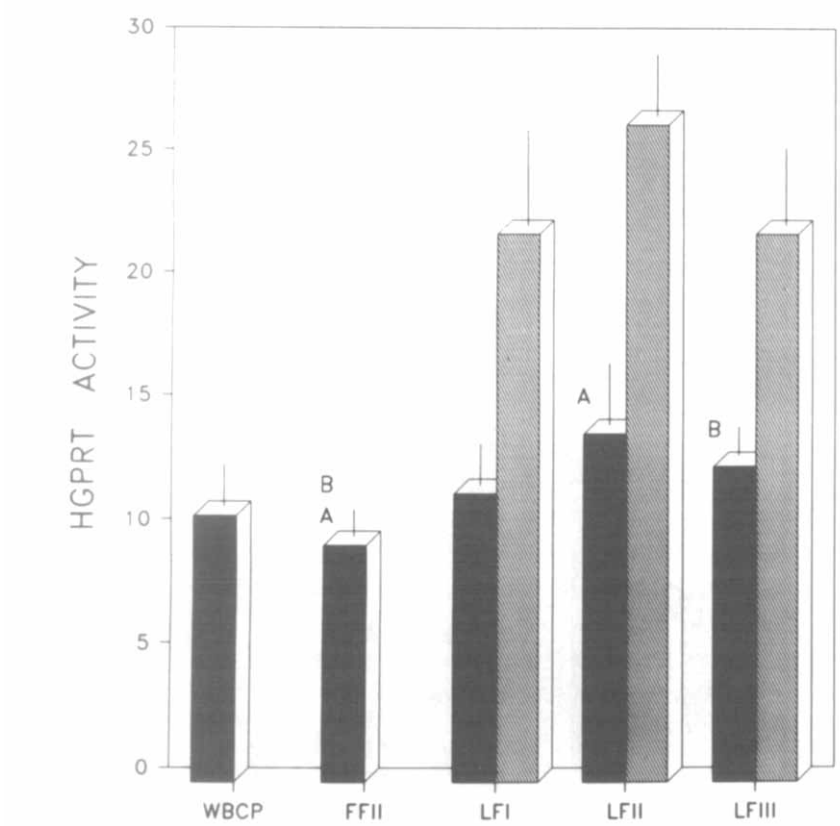

Figure 3. HGPRT activity in the same bursal cell fractions described in Fig. 1. HGPRT-specific activity is expressed as nmoles of product formed/min per mg protein (■) or per 10^8 cells (□). $p < 0.01$ between A; $p < 0.05$ between B.

or are extremely poor in XDH activity. Rapid cleavage of hypoxanthine may actually take place in epithelial cells only in those metabolic conditions in which the intracellular concentration of the purine base cannot be completely and reversibly converted to inosine by PNP, the specific activity of which is six-fold higher than that of XDH, as seen in the FFII. In such conditions, however, a salvaging of hypoxanthine to the corresponding 5'-monophosphates may also occur, although to a lesser extent than its conversion to inosine because of the much lower level of HGPRT in comparison to that of PNP. Therefore, epithelial cells seem to constitutively retain high intracellular levels of inosine and hypoxanthine, the excess of the latter being either irreversibly catabolized to uric acid or recycled to IMP.

By contrast, bursal lymphocytes appear to be particularly able to convert hypoxanthine to IMP since the HGPRT-specific activity was constantly higher than that of XDH and PNP in all the lymphocyte-enriched fractions, especially in the LFIII. Further, virtually all the hypoxanthine produced by PNP in lymphocytes is most likely interconverted to IMP since elimination of uric acid from purine rings does not seem to take place in such cells. The salvaging of hypoxanthine through its transformation into IMP in lymphocytes may well be related to their active intrabursal proliferation (30,31) since the production of newly synthesized nucleotides via purine salvage pathways costs only one molecule of ATP.

Therefore, the main biological role of high levels of HGPRT in bursal lympho-

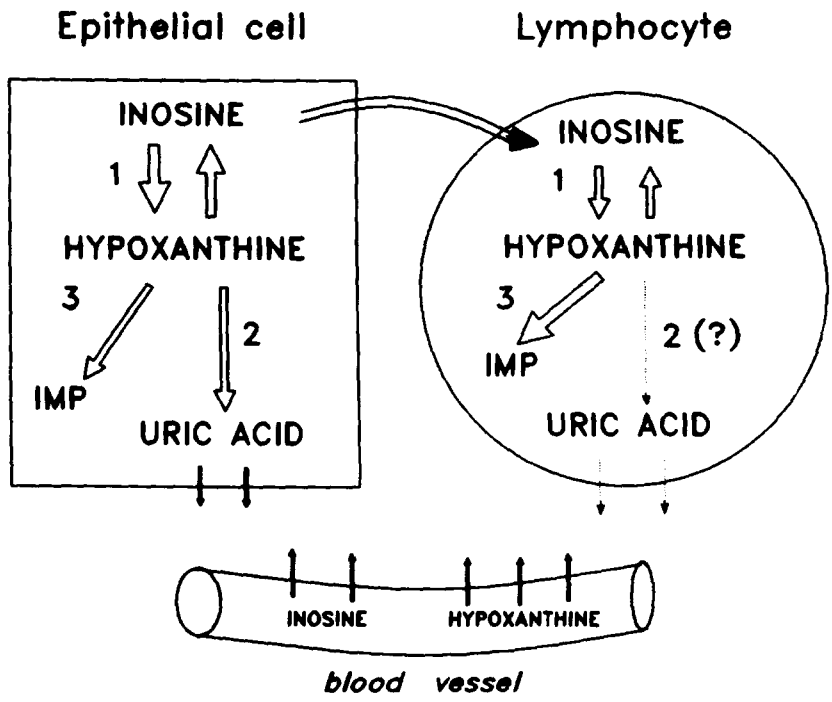

Figure 4. Proposed mechanisms for the purinic metabolic cooperation between bursal epithelial cells and lymphocytes. The wideness of the arrows inside the cells is related to the amount of corresponding enzyme activity. The arrow between the cells indicates the preferential flux of inosine from epithelial cells to lymphocytes. The arrows between the cells and the blood vessels indicate the cell release of uric acid and the release of inosine and hypoxanthine from the bloodstream into the intercellular milieu. (1), PNP; (2), XDH; (3), HGPRT.

cytes appears to be the maintenance of a high rate of purine biosynthesis in such rapidly growing cells by allowing incorporation of hypoxanthine into RNA and DNA instead of its elimination as uric acid (32). The rapid channeling of hypoxanthine to IMP may also account for a lowering of the intracellular concentration of hypoxanthine, which has been recently reported to be cytolytic for lymphocytes, at least in mammals (33). Evidence exists in support of such a critical role for HGPRT in the B-lymphocyte proliferation: Inhibition of HGPRT activity has been proven to affect antibody production in mice (34), and patients with the Lesch–Nyhan syndrome, characterized by HGPRT deficiency, show a subnormal percentage of peripheral blood B lymphocytes, decreased levels of serum IgG, and an impaired proliferative response of lymphocytes to PWM (35).

In view of the continuous flux of hypoxanthine and inosine occurring in the bursa between the bloodstream and cells, the sharply different distribution of the purine metabolic enzymes in lymphocytes and epithelial cells strongly suggests that a biochemical network operates between the two types of cells, allowing a balance of salvaging and degradation of purine bases (Fig. 4). Since lymphocytes lack an efficient mechanism whereby hypoxanthine can be rapidly catabolized to uric acid, any uptake of hypoxanthine from the blood vessels could lead, especially in the cortical lymphocytes, to an increase in the intracellular concentration of this potentially lymphocytolytic purine base, which is present in higher amounts than inosine in

chicken sera (29). Epithelial cells, on the other hand, can prevent the accumulation of hypoxanthine in lymphocytes by draining excess of such a purine base from the bursa microenvironment into the bloodstream. In addition, unlike lymphocytes, epithelial cells possess high levels of PNP by which they can retain high amounts of inosine. Consequently, the possibility exists that lymphocytes take up inosine, which can freely cross the cell plasma membrane, from epithelial cells. The "inosine feeding" of lymphocytes may actively contribute to the high rate of IMP biosynthesis in such cells without increasing the intracellular levels of hypoxanthine. It is tempting to suggest that such a phenomenon is particularly important for medullary lymphocytes, which cannot take up inosine directly from the blood vessels, since there is no vascularization in the medulla of bursal follicles (36,37). Thus, the only route by which inosine might reach medullary lymphocytes from the bloodstream seems to be through the reticular epithelial cells, the processes of which surround medullary lymphocytes and are connected to the corticomedullar epithelial layer (21). Such a layer is separated by a basement membrane from the cortex compartment of the follicle, where, however, an abundant vascularization is present. Eventually, the synthesis of IMP, linked to the uptake of inosine coming from the epithelial cells, might greatly reduce the possibility of an intracellular accumulation of IMP within lymphocytes, thus overcoming the possible functional impairment of such cells due to the potentially toxic effects of high levels of purine nucleotides (10–12).

In conclusion, the physiological role of the metabolic cooperation described might be of relevant importance for the normal development of bursal lymphocytes and provides new insight into the mechanisms by which epithelial cells participate in such a phenomenon.

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