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Pteridine Formation During Lectin-Induced Lymphocyte Activation

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After iodine oxidation, biopterin, 6-hydroxymethylpterin, and 6-formylpterin were identified in mouse spleen lymphocytes by means of reverse-phase HPLC, Crithidia assay, and oxidative degradation. Concanavalin A activation induces a 30-fold increase in the pteridine amounts; biopterin as well as the sum of the carbinol and the aldehyde attain levels of 6–8 $\times$ 10^{-12} mol/10^6 cells. The most rapid increase occurs during the first 24 hr. Thus, pteridine accumulation precedes the period of lymphocyte proliferation; maximum DNA synthesis was found after 72 hr. Biopterin remains largely inside the cells, whereas 6-hydroxymethylpterin and 6-formylpterin were found in the supernatant if the stimulated cells were subsequently incubated in a phosphate buffered salt solution (PBS). Isoxanthopterin was found in the PBS supernatant of control cells that previously were kept in medium alone rather than subjected to lectin stimulation. Only minimal amounts were found inside these cells, and this pterin was absent from the stimulated lymphocytes. The early increase in cellular pteridines and their differential release may well provide the basis for their modulating effect on interleukin-2 activity (Ziegler I, et al: Lymphokine Research 3:284, 1984).

Key words: pteridines, biopterin, 6-hydroxymethylpterin, 6-formylpterin, isoxanthopterin, lymphocyte activation, lectin stimulation, cell proliferation

The production of neopterin by activated T-lymphocytes has been offered as a unifying concept that may explain the increase in urinary neopterin levels that occurs during malignant cases, allograft rejection, and viral infection [1]. The biopterin levels of the mononuclear cells were found to increase some days before clinical symptoms of graft-versus-host reaction were obvious [2]. In vitro experiments were limited to monitoring of neopterin. This pterin was found to be excreted by activated human peripheral blood mononuclear cells into the medium [1]. Recently, macrophages were identified as its immediate source [3]. However, we have no information yet about the range of pteridines occurring in activated lymphocytes, nor are the time kinetics of their formation known.

In previous investigations we had shown that some intermediates of pterin metabolism participate in the control of lymphocyte activation [4–6]. Some metabo-

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lites, eg, sepiapterin and tetrahydrobiopterin, are co-stimulators of lectin-induced T-cell activation [4,5]. The latter pterin enhances the activity of interleukin-2 under certain conditions [6]. Both catabolites isoxanthopterin and xanthopterin inhibit the formation of the 45S pre-rRNA and finally bring about the inhibition of lymphoblast proliferation [7]. These data prompted us to establish the range of pteridines occurring in lymphocytes and to follow the kinetics of their biosynthesis during the course of lectin-induced activation. The modulating effect of some pteridines on interleukin-2 activity and finally on lymphoblast proliferation depends upon the release of the cellular pteridines into the medium. Therefore, this topic was included in our studies.

MATERIALS AND METHODS

Materials

The sources of all compounds used for cultivation and stimulation of lymphocytes were the same as described in [5]. The chemicals that were needed for cell extraction and for the purification steps were analytical grade from Merck (Darmstadt). The Dowex exchange resins were from Serva (Heidelberg). The solvent systems were HPLC grade from Rathburn Chemicals (Walkerburn, Scotland). The pterins were commercially obtained from B. Schircks (Jona, Switzerland). 6-Hydroxylumazine, 6-hydroxymethylpterin, and pterin were a gift from W. Pfleiderer (Konstanz), 7-hydroxylumazine from H. Rembold (München).

Methods

Lymphocyte stimulation and cultivation of the cells. The spleen lymphocytes were prepared from male inbred mice (strain 101, Neuherberg, Germany) aged 7–9 wk. The procedure is described in [5]. The lymphocytes were stimulated by addition of concanavalin A. Its optimum concentration (2.5 μg/ml) had been established by previous experiments. The stimulation was performed in batch cultures of 10–20 ml, which started at a density of 7.5×10^5/ml. The medium composition and all conditions of cell growth were the same as previously described [5].

Harvesting and extraction of the cells. At the periods indicated, 3–5 aliquots (200 μl) were plated onto microtiter wells, and the incorporation of ^{3}H-thymidine was measured [5]. After determination of the viable cell counts, 4–10-ml aliquots were centrifuged at 800g for 5 min, washed with phosphate buffered salt solution (PBS Dulbecco) and kept frozen at −20°C. For studies of the release of pteridines, the separated cells were resuspended in PBS Dulbecco (half of the original culture volume) and kept at 37°C for the periods indicated. Afterward, the cells were separated again by centrifugation and kept frozen at −20°C; the supernatants were lyophilized.

For the kinetic studies during the course of lymphocyte stimulation, the extraction and the iodine oxidation of reduced pteridines were done according to the method described in [8], which was slightly modified. The extraction and iodine oxidation step were performed in 1.5 ml 0.1 N HCl; thereafter, the protein was precipitated by addition of trichloroacetic acid (TCA) (0.5 N final concentration), centrifuged at 10,000g for 30 min, and, in most cases, by additional ultracentrifugation for 2 hr. The resulting supernatant was purified by Dowex H^+ chromatography (Dowex 50

WX 8; 100–200 mesh) as described in [8]. The column size was 35 × 4 mm. Isoxanthopterin, the hydroxylumazines, and the bulk of lumazine were not retained by the Dowex H^+ column. Therefore, the waterwash of the Dowex H^+ column was brought to pH 11 with NH_4OH, and the pteridines in it were purified by Dowex OH^- chromatography (1 × 8, 200–400 mesh). The neutralized eluates of both the anionic and cationic exchange columns were lyophilized. This method recovered 80%–85% of added tetrahydrobiopterin (0.5–1.0 μg in three experiments). A similar recovery is reported by [8]. In pilot experiments the cell extraction was compared with two additional methods. One alternative was extraction with 0.1 N $HClO_4$ and subsequent neutralization with KOH, and the other used steaming for 20 min in 0.1 M KH_2PO_4 with subsequent ultracentrifugation [9]. The extracts were lyophilized. With these procedures, sepiapterin and deoxysepiapterin were sought since recovery experiments had shown their persistence under the conditions prevailing in these methods.

Identification and quantitative determination of the pteridines by reverse-phase HPLC. The following instrumentation assembly was used for the HPLC analysis: Beckman 110 A pump; Altex loop injector (20 μl); Ultrasphere-ODS C 18-column (with a Spherisorb RP 18 Pre-column); and Shimadzu fluorometer Type RF-530 and Shimadzu Integrator C-R1B. The Ultrasphere-ODS column and the pre-column had a particle size of 5 μm. The dimensions were 4.6 × 250 mm. The dimensions of the pre-column were 4.6 × 20 mm. For the blue fluorescent compounds, an excitation wavelength of 350 nm was used, and the emitted light was measured at 450 nm. The corresponding data for the yellow fluorescent pterins are 425 nm/530 nm.

Three different solvent systems were compared: (a) H_3PO_4 (3 mM)/7% methanol/1% acetonitril; (b) 10% methanol; and (c) ammonium phosphate (1 mM) adjusted to pH 7.2. The flow rate was 1 ml/min. The lyophilized cell extracts were dissolved in 80–120 μl H_2O. Thus, the injection of 20-μl aliquots represented the extract of about 0.5–1.0 × 10^6 cells. The fluorescence intensities of their pteridines ranged within linear standard curves that had been set up in the range between 25–1,250 pg.

The lymphocytic pteridines were identified by the following stages. Step one: the retention times of 15 pterins and lumazines were compared in solvent systems a to c; step two: standard pterins and lumazines were co-chromatographed by adding them to the eluates of the ion exchange columns; step three: the ion exchange eluates were fractionated by HPLC in solvent systems a or b. The pteridine fractions from 2–3 runs were lyophilized and individually re-chromatographed with solvent system c. The recovered pteridine fraction was finally co-chromatographed with the standard pteridine under study using the complementary solvent systems b and a, respectively.

For further corroboration of their identity, the pteridines were separated in system a and re-chromatographed in system b. The individual fractions of 4–6 runs were collected and lyophilized. Aliquots were subjected to the Crithidia assay. This bioassay specifically indicates an L-erythro configuration in the side chain at C-6 [10]; the growth-supporting activity of biopterin had been studied in detail in previous experiments [11]. Other aliquots were subjected to $KMnO_4$ oxidation in 0.1 N NaOH. By this procedure, any 6-substituted pterin yields pterin-6-carboxylic acid [12], which was finally identified by HPLC in solvent systems a and b. All analytical procedures were protected from sunlight. Purified pteridine extracts were handled under red light.

RESULTS

Identification of the Pteridines From Mouse Spleen Lymphocytes

The separation of pteridines by reverse-phase HPLC and their standard curves are already well depicted; lists of their retention times are provided [eg, 13]. Therefore, this information will not be repeated here.

A series of pterins and lumazines were ruled out that occur in measurable amounts in resting or in stimulated mouse spleen lymphocytes. They are monapterin, neopterin, 2-amino-4-hydroxypteridine (pterin), 6-methylpterin, xanthopterin, sepiapterin, deoxysepiapterin, lumazine, 6-hydroxylumazine, and 7-hydroxylumazine. Minimal amounts of pterin-6-carboxylic acid (3% maximum of total pterins) are found after controlled iodine oxidation; quantities increased by up to 8% if the cell extraction was performed by perchloric acid or by steam treatment. For this reason, the iodine oxidation method has been used throughout the kinetic studies.

Figure 1 exemplifies a chromatogram of lymphocytic pteridines, which had been retained on the cation exchange column. They were identified as biopterin, 6-hydroxymethylpterin, and 6-formylpterin. Isoxanthopterin was found in the water-wash of this column. Standard pteridines that had been co-chromatographed with the total ammoniacal eluate of the cation exchange column were recovered at their typical positions in either of the solvent systems. Moreover, their amounts (225 pg biopterin, 225 pg 6-hydroxymethylpterin, and 125 pg 6-formylpterin, respectively) added quantitatively to the amounts found in the lymphocytic pterin fractions.

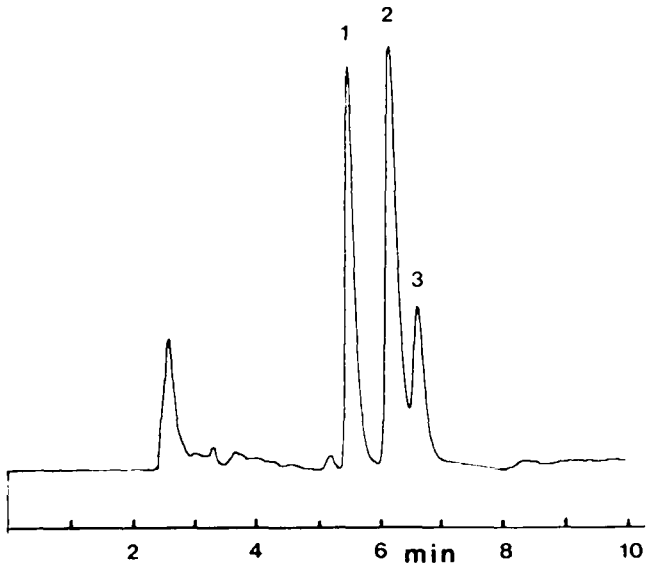

Fig. 1. Elution profile of the Dowex H^+ absorbed pteridines from concanavalin A-stimulated mouse spleen lymphocytes. After acidic extraction and iodine oxidation, the pteridines were purified by the cation exchange column as described in Methods. The neutralized ammoniacal eluate was concentrated by lyophilization and subjected to reverse-phase HPLC with the solvent system H_3PO_4 (3 mM)/7% methanol/1% acetonitril. The fluorescence is determined in arbitrary units. 1) biopterin; 2) 6-hydroxymethylpterin; 3) 6-formylpterin.

Non-fluorescent cellular compounds elute together with pteridines from the Dowex H^+ columns and thus may interfere with the HPLC separation and identification of the pteridines. To minimize their possible effects, the cation exchange column effluent was chromatographed by consecutive runs in two solvent systems. The prefractionated pteridines co-migrated with the standard compounds in the third complementary system and yielded symmetrical peaks.

For further identification, the fraction that emerged at the retention time of biopterin was harvested by five repeated injections of the extract. These fractions were pooled and were subjected to the Crithidia assay. The growth-supporting activity of this fraction corresponded with its fluorometric determination within a range of $\pm 7\%$. The $KMnO_4$ oxidation product of both the 6-hydroxymethylpterin and the 6-formylpterin fractions yielded pterin-6-carbonic acid.

As outlined in Methods, three different procedures for cell extraction were compared. Biopterin, 6-hydroxymethylpterin, and 6-formylpterin were identified by all these methods. However, after steam extraction and after $HClO_4$ treatment, their amounts and their percentage contribution varied considerably, whereas iodine oxidation resulted in largely constant amounts and reproducibile ratios of 6-formylpterin to 6-hydroxymethylpterin; 15%–20% were represented by the aldehyde.

Acetonitril, which is an efficient denaturating component of solvent system a, focuses a bulk of fluorescent fractions that emerge shortly after the front peak (Fig. 1). In solvent systems b and c these fluorescent fractions emerge later and thus may interfere with pteridine analysis. The identity of these substances is under study.

The Lymphocytic Pteridines During Concanavalin A-Induced Activation

From the foregoing results, it is obvious that iodine oxidation in acidic solution, subsequent purification by an ion exchange column, and HPLC analysis with solvent system a was the advisable method for following lymphocytic pteridines during the course of concanavalin A activation. Six complete time courses of lectin stimulation at 24-hr intervals were followed. It is known that the immune responses may vary with individual animals, even though they are of the same age, sex, and nutritional stage [14]. Figure 2 exemplifies an experiment that had resulted in a typical activation rate, as indicated by ^{3}H-thymidine incorporation. It shows that during the first 24 hr, activated but not control cells started to build up reduced pteridines, which yielded biopterin, 6-hydroxymethylpterin, and 6-formylpterin. The latter composed about 15% of the carbinol constantly throughout the activation period. Both the carbinol and the aldehyde behaved as a common pool (see below) and possibly had the same origin (see Discussion). Therefore, their amounts are added to give a single curve in Figure 2. In one experiment, the lymphocytic response was only 10% of the average level, as indicated by the ^{3}H-thymidine incorporation rate. Only a slight concomitant increase in pteridines occurred. In no experiment could isoxanthopterin be traced inside the activated lymphocytes; it was found in only minimal amounts in control cells. It is obvious from Figure 2 that the accumulation of pteridines occurs before the onset of DNA synthesis. This was confirmed in all the activation series performed.

Pteridine Release by Lymphocytes

An efficient lectin stimulation of lymphocytes during a period of 3–4 days presumes their cultivation in a multicomponent medium, eg, RPMI 1640. Fetal calf serum contains biopterin up to a level of 15 ng/ml; 6-hydroxymethylpterin and

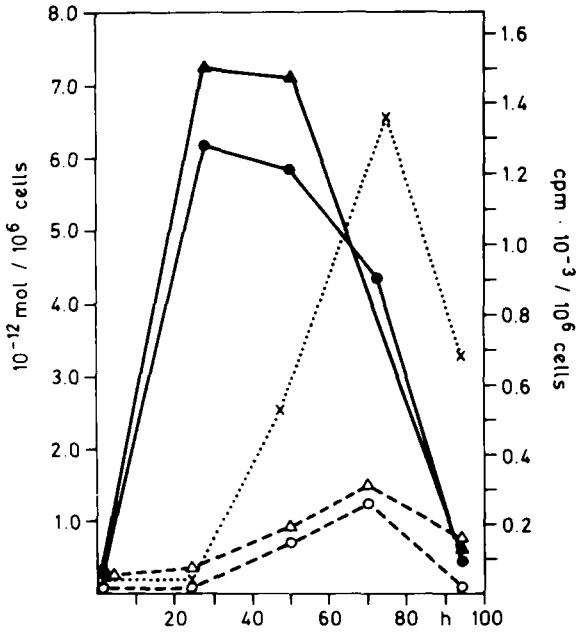

Fig. 2. Time course of pteridine accumulation in mouse spleen lymphocytes that were activated by concanavalin A (2.5 μg/ml). After acidic extraction and iodine oxidation, the cellular pteridines were purified by the cation exchange column as described in the Methods. The neutralized ammoniacal eluate was concentrated by lyophilization and subjected to reverse-phase HPLC with the solvent system H_3PO_4 (3 mM)/7% methanol/1% acetonitril. (○- - -○), biopterin from controls; (△- - -△), 6-hydroxymethylpterin + 6-formylpterin from controls; (●——●), biopterin from stimulated cells; (▲——▲), 6-hydroxymethylpterin + 6-formylpterin from stimulated cells; (×.....×), incorporation of ^{3}H-thymidine.

6-formylpterin, besides some degradation products, are present at varying concentrations (unpublished results). This complicates the analysis of pteridines that are released from the activated lymphocytes into the culture medium. For this reason, the cells were incubated in PBS Dulbecco after they had been activated in the complete culture medium and the released pteridines were analyzed. The biopterin portion was largely retained inside the cells, as is illustrated in the case of 72-hr stimulated lymphocytes (Fig. 3) whereas the 6-hydroxymethylpterin + 6-formylpterin were recovered from the supernatant soon after resuspension. Both the remaining and the supernatant portions added up to about 95% of the amounts for non-incubated cells. Glucose (0.1%) or Ca^{++} (0.9 mM) + Mg^{++} (0.5 mM) or both did not appreciably change the rate of release.

The capacity for pteridine release by control cells and during the course of lectin stimulation is illustrated in Figure 4. It is obvious that the release of 6-hydroxymethylpterin + 6-formylpterin parallels their amounts built up in the activated cells (see Fig. 2). In contrast, the marked increase in cellular biopterin was not matched in the medium.

Isoxanthopterin was below the detection limits inside the activated cells. It can be traced in small amounts in their supernatants (Fig. 4, right side). The small amounts of isoxanthopterin released from these stimulated cells may be responsible for the 3%–6% lacking in the HPLC pattern of the Dowex H^+ absorbed pteridines

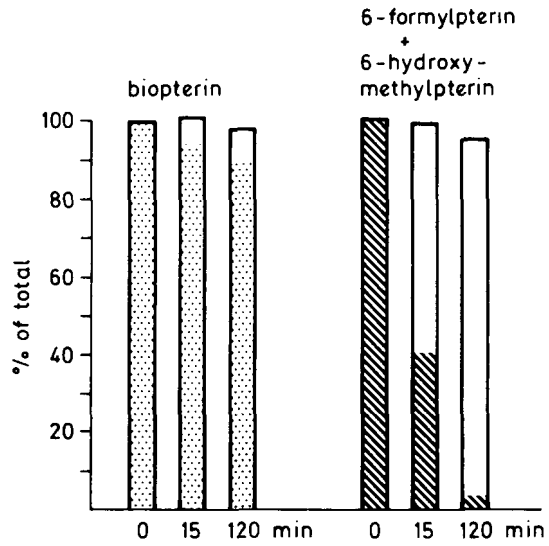

Fig. 3. Release of pteridines during resuspension of lectin-stimulated mouse spleen lymphocytes. The lymphocytes had been stimulated for 72 hr by concanavalin A (2.5 μg/ml) and were then resuspended in PBS Dulbecco for the time periods indicated. After acidic extraction of the cells and after iodine oxidation of both the cells and the supernatant, the pteridines were purified by the cation exchange column as described in Methods. The neutralized ammoniacal eluate was concentrated by lyophilization and subjected to reverse-phase HPLC with the solvent system H_3PO_4 (3 mM)/7% methanol/1% acetonitril.

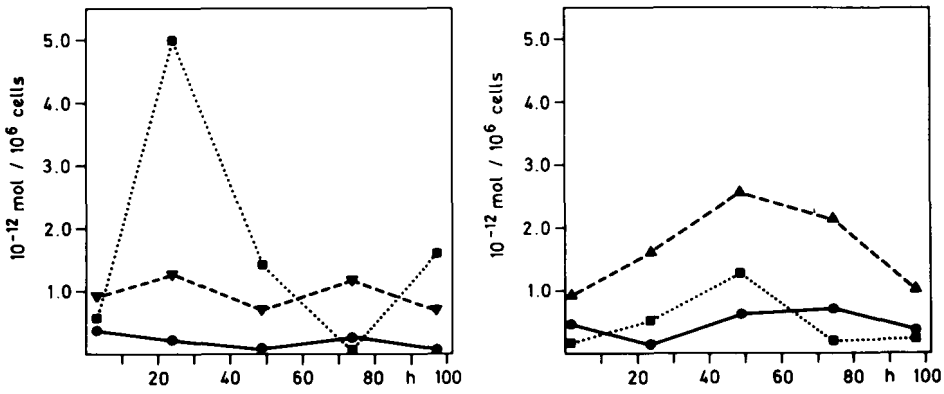

Fig. 4. Left side: release of pteridines by control mouse spleen lymphocytes. The cells were cultivated in medium during the periods indicated and then suspended for 2 hr in PBS Dulbecco. The supernatant was processed as described in the legend to Figure 3. Right side: release of pteridines by lectin-stimulated mouse spleen lymphocytes. The lymphocytes had been stimulated by concanavalin A (2.5 μg/ml) during the periods indicated and then suspended for 2 hr in PBS Dulbecco. The supernatant was processed as described in the legend to Figure 3. (●——●), biopterin; (▲- - - -▲), 6-hydroxymethylpterin + 6-formylpterin; (■.....■), isoxanthopterin.

(see Fig. 3). Controls, however, that previously were kept in medium alone rather than subjected to lectin stimulation consistently showed a marked though variable ($2.5–5.0 \times 10^{-12}$ mol/10^6 cells) isoxanthopterin release during the first period of their incubation in the medium. From the data presented above, it is calculated that under our experimental conditions, these cells may render the medium up to 6×10^{-9} M with respect to isoxanthopterin.

Injection of the PBS supernatant, after appropriate concentration, was tried directly into the HPLC system, as is usually done with biological fluids, eg, urine [15]. However, peaks were broad, poorly separated, and followed close behind the front. Only processing of the PBS supernatant in the same way as the cells yielded clear HPLC patterns as described above.

DISCUSSION

The experiments illustrate for the first time that cellular pteridines are accumulated during in vitro stimulation of mouse spleen lymphocytes and identify biopterin, 6-hydroxymethylpterin, and 6-formylpterin as their oxidation products. Unlike humans [1,15], mice do not produce neopterin. The buildup of pteridines is an early event during lymphocyte stimulation and clearly precedes the DNA synthesis of the activated cells. Thus, the pteridine formation coincides with the period of interleukin-2 production [cf 16]. This may provide the basis for the modulating effect of tetrahydrobiopterin on the activity of this lymphokine [6] and finally result in the enhancement of lymphocyte proliferation [4,5].

Our experiments analyze the total of mouse spleen lymphocytes and do not differentiate between the various types of mononuclear cells. Cellular differentiation was achieved with respect to neopterin release in humans. It was attributed to the macrophages [3]. The analysis of the complete range of pteridines and their assignment for the various types of mononuclear cells in both humans and in mice is under study.

The experiments indicate that three pteridine pools may be built up in lymphocytes. The first one leaves biopterin inside the cells after iodine oxidation; the second gives rise to 6-hydroxymethylpterin and 6-formylpterin and is released by the stimulated cells; the third results in isoxanthopterin, which is found as an excretion product in the supernatant. The process that triggers the formation of this pterin in cultured cells in absence of a mitogen is as yet unknown. It may be produced during the decay of unstimulated cells. The results point to the view that the catabolic steps either occur at the outer membrane or are just simply brought about by enzymes that are simultaneously released with the precursor. Xanthine oxidase is known to introduce an oxygen function into both positions 6 and 7 of the pteridine ring [17]. Since this enzyme may hold a key function in pterin catabolism and the data on its activity in lymphocytes are controversial [18,19], a thorough investigation is needed.

According to the current view, polyhydroxyalkyl substituted pteridines cannot be derived from folic acid catabolism. Thus, it safely can be assumed that the increase in lymphocytic biopterin results from de novo synthesis. Pteridines, which are substituted at C-6 by a one-carbon unit, however, may be derived from folate degradation under the conditions experienced during our analyses or originate from folate catabolism of the cells. Recovery experiments with various folates and reduced

folates made it most unlikely that the 6-hydroxymethylpterin was derived from their chemical degradation (experiments not shown in detail). A conversion of folate into the aldehyde and the carbinol, respectively, by malignant cells has been claimed [20,21] but not corroborated further. Thus, the origin of both 6-hydroxymethylpterin and 6-formylpterin in activated lymphocytes awaits further clarification.

Mice bearing Ehrlich ascites tumor excrete urinary 7,8-dihydro-6-hydroxylumazine [22]. No lumazine derivative could be found in the stimulated mouse spleen lymphocytes nor were any released by the cells. Therefore, the 6-hydroxylumazine must be a product from the ascites cells or, more likely, deamination of pteridines occurs only during further metabolism, eg, in the liver.

By way of the immune system, the pteridine accumulation can be triggered via the immunogenic stimulus, eg, the lectin or the antigen. This facilitates kinetic studies of their synthesis. However, increased cellular biopterin levels also were found in other systems, eg, during hemopoietic cell proliferation [11] and during proliferative disorders of any of the blood cell lineages [23]. There are early observations that tetrahydrobiopterin temporarily piles up in the regeneration bud of amphibians [24], and 6-hydroxymethylpterin was found in transformed cell lines [21]; an acrasin of Dictyostelium lacteum was more recently identified as a pterin [25]. In these systems the kinetics of pteridine accumulation are largely unknown.

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