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The immune system of the trout and other teleosts exhibits two striking anatomical differences from mammals: 1) they possess neither bone marrow nor lymph nodes; and 2) they harbor extensive immune tissue within the kidney (1). Despite this unique locale of lymphoid tissue, fish are able to execute immune functions comparable to those observed in mammals. For example, the lymphopoietic/hematopoietic functions of the mammalian bone marrow appear to occur primarily in the teleost anterior kidney (1, 2). Also, the teleost anterior kidney produces many of the same factors, such as Ikaros (3), RAG-1 and -2 (4, 5), and TdT (6), that are used for mammalian lymphocyte maturation.

Recent reports have indicated that there are functionally unique subpopulations of Ab-secreting cells (ASC) present within mammals, including the plasmablast (7, 8), the short-lived plasma cell (9, 10), and the long-lived plasma cell (11–15). The plasmablast is an ASC that is distinguished from plasma cells by its ability to proliferate, its short half-life, and its low levels of CD138, B220, and presence of c-Myc (7, 16–18). In mammals it appears that much of the early Ab response is dominated by plasmablasts, which are located primarily within the peripheral immune organs (19, 20). Plasmablasts are thought to undergo rapid clonal expansion, leading to the generation of a large population of terminally differentiated, short-lived plasma cells in the periphery (8).

Plasmablast and Plasma Cell Production and Distribution in Trout Immune Tissues

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These studies describe the in vitro and ex vivo generation of plasmablasts and plasma cells in trout (Oncorhynchus mykiss) peripheral blood and splenic and anterior kidney tissues. Cells were derived either from naïve trout and cultured with the polyclonal activator, Escherichia coli LPS, or from trout that had been immunized with trinitrophenyl-keyhole limpet hemocyanin. Hydroxyurea was used to resolve populations of replicating (plasmablast) and nonreplicating (plasma cell) Ab-secreting cells (ASC). Complete inhibition of Ig secretion was only observed within the PBL. Both anterior kidney and splenic lymphocytes possessed a subset of ASCs that were hydroxyurea resistant. Thus, in vitro production of plasma cells appears to be restricted to the latter two tissues, whereas peripheral blood is exclusively restricted to the production of plasmablasts. After immunization with trinitrophenyl-keyhole limpet hemocyanin, specific ASC could be isolated from all immune organs; however, the anterior kidney contained 98% of all ASC. Late in the response (>10 wk), anterior kidney ASC secreted specific Ab for at least 15 days in culture, indicating that they were long-lived plasma cells. Cells from spleen and peripheral blood lost all capacity to secrete specific Ab in the absence of Ag. Late in the Ab response, high serum titer levels are solely the result of Ig secretion from anterior kidney plasma cells. The Journal of Immunology, 2004, 173: 7317–7323.

Plasma cells may become long-lived upon migration to a supportive niche within the bone marrow, relying on specialized cues for their longevity (14). These long-lived plasma cells continually produce Ab for months to years in the absence of stimulating Ag and are pivotal to the long term protection of a mammalian host against invading pathogens (21–23). The discovery of long-lived plasma cells in the bone marrow of mammals has significantly altered the view of immunological memory (11, 13, 14). This preservation of plasma cell activity results in what has been termed humoral memory (13). This differs from the classical memory paradigm in that it relies on the maintenance of Ab titers over a significant proportion of the host’s life, while not requiring antigenic restimulation (11, 12).

To date, little work has focused on ASC diversity in teleosts. The extent of current research has been limited to the generation of ASCs, which have been collectively described as plasma cells (24–28). These plasma cells can be isolated from the three major immune tissues in fish: spleen, peripheral blood, and kidney (29–31).

The assumption that teleosts (or any other species) possess only one type of ASC, the plasma cell, dramatically influences models of functional organization of the teleost immune system. Recognition of subpopulations of ASC and their functions/distribution is essential to devising a more cogent approach to modeling immune system functions. Additionally this may provide intriguing insights into the evolutionary development of the immune system, lymphocyte trafficking, and immunoprophylaxis. Thus, in this study the dynamics of activated B cell proliferation and Ig secretion in rainbow trout were explored. In particular, the differential response patterns observed in leukocytes isolated from various immune tissues were examined and characterized.

Materials and Methods

Animals

Three-year-old rainbow trout were maintained in 300-gallon tanks within a recirculating system using biologically filtered, dechlorinated, chemically balanced, and UV-treated city water. Fresh water exchange was ~4%/day, with 75% of the volume recirculated through the biofilter per hour. Water
temperature was maintained at 10°C, and photoperiod was adjusted to match seasonal change. Fish were fed dry pellet feed (ASD2; Ziegler Brothers, Gardners, PA).

**Cell culture**

Trout leukocyte isolation and culture were modifications of the procedure described by Yui and Kaattari (32). Briefly, peripheral blood cells were pelleted by centrifugation at 500 × g (4°C) and resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) to a total of 8 times the original blood volume. Anterior kidney and splenic tissues were teased apart with sterile bent Pasteur pipettes, and a cellular suspension was achieved by repeated aspirations through a 5-ml syringe using a 22-gauge needle. Anterior kidney and splenic cell suspensions were diluted in RPMI 1640 (In Vitrogen Life Technologies, Gaithersburg, MD) to 48 ml/organ. Twelve milliliters of each tissue suspension was layered upon an equal volume of Histopaque 1077 (Sigma-Aldrich) in 50-ml conical centrifuge tubes (BD Biosciences, San Jose, CA) and centrifuged (500 × g) for 40 min. Leukocytes collected from the interface layer were washed three times by centrifugation in RPMI 1640, viability was determined by trypan blue, and cells were resuspended to a concentration of 2 × 10^7 cells/ml in tissue culture medium (TCM) (33). Aliquots of 50 μl of the cell suspension were distributed into each well of a 96-well, cell culture cluster plate with a low evaporation lid (Corning, Corning, NY). Either 50 μl of Escherichia coli LPS serotype 055:B5 (200 μg/ml; Sigma-Aldrich) or TCM (control) was added to each well (34). Starting on the day after initiation of cell culture, 10 μl of feeding mixture supplement (34) was added on alternate days. Plates were placed into an incubator culture chamber supplied with blood gas and incubated at 17°C.

**Hydroxyurea (HU) inhibition**

HU experiments were performed to assess the possible linkage between proliferation and ASC production, thus functionally distinguishing between HU-sensitive (plasmablast) and HU-insensitive (plasma cell) activities. The optimum HU concentration for cellular proliferation arrest in trout lymphocytes was determined to be 100 mM, which was achieved by adding 10 μl of a 1 M solution in TCM to each well on the indicated days.

**ELISPOT analysis**

ASC were assessed using a modification of a previously devised ELISPOT assay (35). Briefly, polyvinylidene difluoride membranes were membranes were incubated in methanol, allowed to soak in Milli-Q water, backed with Parafilm, and inserted into a dot-blot apparatus (Bio-Rad, Hercules, CA). Fifty microliters of a 1 μg/ml solution of anti-trout Ig mAb 1–14 (0.5 μg/ml; Sigma-Aldrich) or TCM (control) was added to each well (34). Starting on the day after initiation of cell culture, 10 μl of feeding mixture supplement (34) was added on alternate days. Plates were placed into an incubator culture chamber supplied with blood gas and incubated at 17°C.

**Immunization and temporal assessment of ASC and Ab titers**

One hundred trout (150 g) were immunized with TNP-KLH in CFA and monitored over an extended period for the expression of anti-TNP-specific plasmablasts and plasma cells in peripheral blood, spleen, and anterior kidney. At regular postimmunization intervals, lymphocytes from these tissues were directly assessed for anti-TNP ASC by ELISPOT or were cultured in the continual presence of HU over a period of 14 days to assess the life span of these ASC. The anti-TNP titers from sera were determined by an Ag-capture ELISA (37).

**FIGURE 1.** In vitro polyclonal activation of ASC from peripheral blood, spleen, and anterior kidney. Cultures of PBL, spleen, and anterior kidney generated substantial ASCs upon exposure to LPS (triangles) in vitro compared with control cultures (circles). The mean and SEM for the normalized values from six independent experiments are given. The average ASC maxima were 452/10^6 leukocytes (PBL), 309/10^6 (spleen), and 1435/10^6 (anterior kidney).
Results

Kinetics of ASC production

LPS induced significant ASC responses in PBL, splenocytes, and anterior kidney lymphocytes (Fig. 1). All responses progressed significantly above background after 4 days of culture. Also of particular interest was the observation of an early spontaneous ASC response with the anterior kidney and, to a lesser extent, with the spleen.

$^3$H uptake typically preceded ASC production; however, a general increase in $^3$H uptake was also observed in unstimulated controls (data not shown). The latter probably reflected spontaneous non-B cell proliferation.

Effect of HU on proliferation and ASC function

The timed addition of HU allowed for the resolution of the proliferative phases that are required for differentiation into ASC within each tissue (Fig. 2). The addition of HU to all cultures resulted in complete cessation of $^3$H uptake (proliferation) within 24 h for all cultures (data not shown). Addition of HU to cultures early (days 0–4) uniformly terminated all ASC development within the peripheral blood, but not within anterior kidney or spleen. The kinetics of HU-mediated suppression also revealed that most LPS-inducible ASC (>5 days) from peripheral blood are completely inhibited by HU within 24 h of addition. However, a subset of LPS-induced ASC in the anterior kidney and spleen continued to secrete Ig for prolonged periods of time.

Kinetics of HU-mediated inhibition of proliferation and ASC function

Compared with other organs, the bulk of the ASC response generated from peripheral blood appeared to be dependent on proliferation. A kinetic study was conducted to achieve a more precise resolution of this temporal linkage. A day 7 culture of LPS-stimulated PBLs was treated with HU at intervals of 48, 24, 12, 8, 6, 4, or 2 h before harvest. This experiment demonstrated that within 24 h of harvest, addition of HU could completely inhibit $^3$H uptake (Fig. 3). When HU was added at times closer to the harvest, the response was proportionately lessened, closely paralleling the degree of ASC inhibition.

Characterization of the ex vivo ASC response

Peripheral blood, spleen, and anterior kidney were assessed periodically for the presence of Ag-specific ASC over a period of 35 wk after immunization. Fish were immunized with TNP-KLH (Fig. 4), and the numbers of anti-TNP ASC were determined at various times postimmunization. Eight weeks postimmunization all organs exhibited a peak of TNP-specific ASC, with both spleen and PBL demonstrating a subsequent decline to undetectable levels. In contrast, the anterior kidney continued to produce high numbers of Ag-specific ASC throughout the test period of 35 wk.

Additionally, at each time point leukocytes from blood, spleen, and anterior kidney were cultured in the absence of Ag, but in the presence of HU, to determine specific ASC longevity. Early in the response (wk 3), ASC from all tissues were HU-sensitive; however, after 10 wk, ASC were only found in the anterior kidney. These ASC were HU-resistant (plasma cells) and continued to secrete Abs for 15 days in vitro (Fig. 5).

Examination of ELISPOT morphology also revealed striking differences among the ASC from the various tissues (Fig. 5). The size of the ELISPOTs ranged from 0.01–1.0 mm, with the average size being ~0.04 mm. Large (>0.2 mm) ELISPOTs were found only in the early response of the spleen, but in anterior kidney they accounted for 3% of the total ASC response throughout the entire culture period. Intensely staining ELISPOTs were primarily associated with the anterior kidney and, to a lesser degree, with the spleen. The specific serum Ab titers of immunized fish increased minimally in the first 10 wk postimmunization (increasing from 1,000 to 3,000 U/ml). After this period, a more significant increase was observed, with serum Ab titers increasing from 3,000 to 75,000 U/ml.

FIGURE 2. The relationship between the addition of HU and the generation of ASC. HU was added at various time points (arrows) to peripheral blood, spleen, and anterior kidney lymphocytes that had been stimulated with LPS on day 0. Each arrow and line originating from that point (alternating solid and dashed lines for clarity) depict the response generated subsequent to the addition of HU to that culture.

FIGURE 3. The relationship between plasma cell generation and cellular proliferation of PBL. HU was added at various times before the harvest of PBLs, and $^3$H uptake (dashed line, □) and number of ASC (solid line, ♦) were assessed.

FIGURE 5. The size distribution of ELISPOTs from peripheral blood, spleen, and anterior kidney lymphocytes. The ELISPOTs were stained with anti-IgG antibodies and visualized under a microscope. The size of the spots was recorded, and the data were plotted. The graph shows the distribution of ELISPOT sizes (0.01–1.0 mm) for each tissue. The specific serum Ab titers of immunized fish are also shown, with minimal increase in the first 10 wk postimmunization and a significant increase thereafter.
Discussion

The ASC response was kinetically characterized in PBL, spleen, and anterior kidney, because these tissues represent distinct primary and secondary lymphoid tissues within the trout. Thus, functional B cell heterogeneity, if comparable to that of mammals, could be distinguished by analyses of these tissues. Specific induction of the ASC response was fairly comparable between tissues, with peak responses occurring by day 7 and possibly a bimodal response in PBL and spleen. Although our statistical analysis demonstrates that this bimodality is significant and repeatable, we are not certain of its origin (i.e., a reflection of B cell population dynamics or simply a culture artifact). Interestingly, spleen and particularly anterior kidney demonstrated a large ASC response in the absence of LPS on the first day of culture (Fig. 1). The possible source of these spontaneous ASC may be previously located within the murine periphery (43).

B cells, i.e., plasmablasts, as is the case with Ig-secreting cells and ASC secretion (G1) are closely situated temporally. This is in agreement with mammalian observations, where Ig secretion occurs in late G1 (40–42). These data conclusively demonstrate that 1) PBL undergo a complete cell cycle in 24 h; and 2) the phases in the cell cycle involved with proliferation (S phase) and ASC secretion (G1) are closely situated temporally. This is in agreement with mammalian observations, where Ig secretion occurs in late G1 (40–42). These data conclusively demonstrate that the entire Ig response within PBLs is produced by actively cycling B cells, i.e., plasmablasts, as is the case with Ig-secreting cells located within the murine periphery (43).

The high degree of spontaneous Ig secretion and proliferation in trout anterior kidney has also been previously reported in another teleost, Limanda limanda (38). The cause of this phenomenon is not yet fully understood; clearly, the presence of a multiplicity of developing hemopoietic cells may explain the high levels of proliferation encountered (39). Additionally, the spontaneous appearance of ASC in the primary cultures may indicate that some low, but significant, number of ASC may persist from previous antigenic exposures.

The addition of HU to LPS-stimulated lymphocytes uniformly terminated proliferation (³H T uptake) in lymphocytes isolated from all immune tissues (data not shown). This treatment also resulted in the complete cessation, within 24 h, of ASC activity within the peripheral blood (100% reduction), 70% reduction in the anterior kidney, and ~50% reduction in the spleen. These results demonstrate that although all tissues produce ASCs, those from the peripheral blood become exclusively replicating ASC, or plasmablasts. The data also reveal that late in the splenic and anterior kidney responses, populations of HU-resistant ASC arise. Because these cells are HU insensitive and are necessarily non-replicating, they must represent a population of plasma cells. These data suggest that normal peripheral blood possesses mature naive B cells, which cannot generate and maintain a population of plasma cells. In contrast to peripheral blood, spleen and anterior kidney are able to generate both plasmablasts and plasma cells. The latter is presumably due to a microenvironment supportive of plasma cell maintenance and survival (13, 14).

To further explore the dependency of plasmablast Ig secretion on proliferation, the staggered addition of HU to PBL was examined over a 48-h period. Proliferation and ASC production temporally paralleled each other, indicating the dependency of ASC proliferation. Specifically, the addition of HU 24 h before harvest completely arrested cellular proliferation (Fig. 3), whereas the addition of HU at later time points resulted in a parallel reduction in both proliferation and ASC generation. Together these data suggest that 1) PBL undergo a complete cell cycle in ~24 h; and 2) the phases in the cell cycle involved with proliferation (S phase) and ASC secretion (G1) are closely situated temporally. This is in agreement with mammalian observations, where Ig secretion occurs in late G1 (40–42). These data conclusively demonstrate that the entire Ig response within PBLs is produced by actively cycling B cells, i.e., plasmablasts, as is the case with Ig-secreting cells located within the murine periphery (43).

In vitro production of HU-resistant ASC within anterior kidney and spleen together with the spontaneous appearance of ASC from naïve hosts suggest a differential distribution of ASC within trout immune tissues. These observations prompted the in vivo examination of ASC distribution postimmunization. This goal was accomplished by immunizing trout with TNP-KLH and monitoring the expression of anti-TNP-specific ASC in blood, spleen,
and anterior kidney over an extended period. Initially, all tissues demonstrated an ASC response that peaked at 8 wk (Fig. 4). After this peak response, ASC in the peripheral tissues (blood and spleen) fell to undetectable levels, whereas the anterior kidney maintained a relatively high response until the end of the test period (35 wk). Even at this latter time point, the responses in anterior kidney did not show signs of diminishing. The persistence of ASC in anterior kidney suggest that auxiliary, or stromal-like, tissue supports plasma cell maintenance, as found in mammalian bone marrow (14).

Examination of ASC from immune tissues of immunized trout revealed that the majority of ASC generated early in the response were HU sensitive. However, virtually all anterior kidney ASC occurring late in the response (>10 wk) were HU-resistant and produced Ab in culture for at least 15 days without mitogenic stimulation, indicating that these cells were long-lived plasma cells. Importantly, the production of a significant serum titer was linked to the appearance and maintenance of these cells within the anterior kidney (Fig. 4). The association of serum titers with ASC numbers in the teleost peripheral tissues has received minimal attention (26, 44), and there is no available literature that explores the association of serum titer with anterior kidney ASC. However, the observed relationship between the increased serum titer and the production of anterior kidney plasma cells prompts speculation on the Ig secretion rate of plasmablasts vs plasma cells. Published observations of the murine system indicate that plasma cells produce significantly greater amounts of Ab than observed with the early HU-sensitive ASC (plasmablast) response. The presence of plasma cells in the anterior kidney is also supported by the size and intensity of the ELISPOTs (Fig. 5). Together, these observations strongly suggest that the primary lymphoid tissue, anterior kidney, harbors long-lived plasma cells. Also, the anterior kidney appears to play the key role in maintaining Ab titers in trout, as witnessed by the correlation with the ex vivo ASC response (Fig. 4). Whether such cells arise from precursors within the anterior kidney or whether they traffic to this organ from the periphery, as has been hypothesized to occur in mammals (49), has yet to be addressed.

Parenthetically, it could be concluded from our data that in vitro culture of peripheral blood and splenic lymphocytes should not be used as a model of all in vivo ASC function. Specifically, if antigenic stimulation of PBL results in the short term production of plasmablasts, the conclusions drawn from such experiments can only, at best, be related to the initial Ab response (wk 0–3) and would have limited bearing on the in vivo generation of long term specific Ab production. It is intriguing in this regard that a teleost peripheral blood B cell line isolated by Miller et al. (50) possessed characteristics of a plasmablast (18-h cell cycle, low Ig secretion rates, and coexpression of sIgM). Our studies suggest that peripheral blood B cell clones may primarily possess this profile (plasmablasts), as opposed to clones derived from anterior kidney or spleen.

A model has been developed from the data presented and from previously published work on ASC development in rainbow trout (Fig. 6). The anterior kidney has been implicated as a major site of B cell development and maturation in adult teleosts (4, 51). From this tissue, mature naive B cells migrate via peripheral blood into other immune tissues, such as the spleen. Once mature naive B

![FIGURE 6. Proposed model for plasmablast/plasma cell distribution within anterior kidney, spleen, and blood. The anterior kidney is posed as the major site of B lymphocyte development. Mature, naive B cells (a) arise within the anterior kidney and are distributed to peripheral tissue via the blood. These B cells encounter Ag and mature into plasmablasts (b) or plasma cells (c) in either the anterior kidney or periphery. Plasmablasts or plasma cells differentiating within the periphery home to the anterior kidney, wherein plasma cells from all tissues compete for long term maintenance within the survival niches of the anterior kidney.](http://www.jimmunol.org/)
cells encounter Ag in immune tissues, they differentiate into plasmablasts, accounting for the initial humoral immune response observed. After successive rounds of proliferation (52), the plasmablasts may traffic from the periphery (i.e., spleen) to the anterior kidney and terminally differentiate into plasma cells (8, 52). Upon receiving specialized cues (53, 54), plasma cells may home and situate themselves within anterior kidney survival niches (13, 14), which foster their long-term survival. Additionally, the total number of lymphocytes that can be recovered from the anterior kidney greatly exceeds that available in the peripheral blood and spleen (ratio of 10:4:1, respectively; our unpublished observations). Thus, it is possible that the differentiation of anterior kidney plasmablasts to plasma cells may be responsible for virtually all the long-lived plasma cell production observed in this organ.

This work demonstrates that ASC can be functionally differentiated into plasmablasts and plasma cells. These cell populations are not uniformly distributed in trout lymphoid organs, with a distinction observed between the lymphocytes located in peripheral tissues and those in central lymphoid tissue. The discovery of distinct populations of ASC in the rainbow trout has many implications for the design and delivery of vaccines. Successful vaccination may require long-term maintenance of plasma cells within the anterior kidney. Considerable work demonstrates variability in the duration of Ab responses by a variety of Ags, perhaps indicating that the ability to specifically generate a long-term response may be due to the physiologic environment of the anterior kidney to supply a suitable niche. A thorough understanding of how to facilitate the production of this niche, via endocrine or pharmacological manipulation, may lead to our ability to enhance vaccine effectiveness.

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