which distinguish *P. falciparum* strains in addition to the isoenzyme (*12, 13*), protein variants (*14*), and S antigen (*8*) markers already in use. Since most of the strain-specific determinants identified in this work are associated with the surface of the merozoite, it will be of interest to ascertain the function of the antigens in protective immunity. Should they prove to be important in this respect, it would obviously be necessary to determine the full extent of the antigenic diversity and its genetic basis, in order to assess the potential effectiveness of a vaccine based on such antigens.

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Hatching of Amphibian Embryos: The Physiological Trigger

Abstract. *Marbled salamander embryos hatch in water if the environmental oxygen pressure is 86 torr or less, but do not hatch if the environmental oxygen pressure is equivalent to that of air. Under hypoxic conditions, embryos hatch in aqueous and nonaqueous media with equal success. Increasing carbon dioxide pressure does not induce hatching, but does decrease the time to hatching by altering environmental* pH.

Prior to hatching, virtually all anuran and urodele embryos develop numerous unicellular ectodermal glands containing hatching enzymes (*1, 2*). Hatching involves the extracellular release of these enzymes, which digest the vitelline membrane and gelatinous egg capsule. After the egg capsule is partially digested, the embryo completes hatching by muscular activity. Although there are numerous descriptions of hatching (*3*) the environmental or physiological factors that initiate enzyme release and subsequent hatching are unknown.

The marbled salamander *Ambystoma opacum* deposits eggs in terrestrial nests in the depressions of temporary pools or along the margins of reduced pools (*4*). Within a few weeks, the embryos develop to a stage capable of hatching and then remain quiescent within the egg capsule until the nest is flooded by rainwater. Embryos may remain at that stage for months within the egg capsule, but typically hatch within minutes to hours after they are covered by water. This curious phenomenon prompted us to investigate what factors initiate hatching in this species. Earlier workers noted that submerging marbled salamander eggs in water stimulated embryos to hatch and implied that water played an essential role in the hatching process (*5*).

Experiments in which we varied either the medium or the composition of gases surrounding the eggs (*6*) indicate that a reduced oxygen pressure (PO_2), rather than water per se, is the trigger for hatching (Table 1). In water (rows 1 to 4) hatching occurred only when the PO_2 was below that of the atmosphere (about 150 torr). The effects of PO_2 on hatching time were determined by either allowing embryos to consume oxygen in a closed system (row 1) or by lowering ambient PO_2 experimentally (row 4). In both cases embryos hatched. In contrast, hatching did not occur when embryos were maintained in an environment in which PO_2 was at or above atmospheric levels (rows 2 and 3). In an initial trial, 2 of 12 embryos hatched in aerated water; however, this occurred during failure of our aeration system which presumably resulted in a low PO_2. In later trials with more than 200 embryos, hatching did not occur when embryos were maintained in aerated water. Embryos that were placed in water equilibrated with 100 percent O_2 (row 3) did not hatch even after 10.5 hours; however, when transferred to water equilibrated with 100 percent N_2 they hatched rapidly ($\bar{x}$ = 23 minutes, row 4). Embryos also hatched in cooking oil (row 5) and in gaseous nitrogen (row 6). These results demonstrate that external water is not essential for hatching and support our contention that low PO_2 triggers hatching.

Carbon dioxide did not induce hatching when PO_2 was at or above atmospheric levels (rows 2 to 5 in Table 2). Hatching occurred more rapidly when water was equilibrated with 5 or 10 percent CO_2 in nitrogen (rows 9 and 10) than when equilibrated with 100 percent nitrogen (rows 6 to 8). At 23°C, embryos exposed to elevated CO_2 hatched about

Table 1. Effects of hypoxia on the hatching of *A. opacum* embryos. In all experiments, groups of four embryos were placed at room temperature (22° to 24°C) into vials (5.3 by 2.5 cm) fitted with inflow and outflow valves. Eggs in water were either allowed to stand without agitation, or were gently agitated with air, O_2, or N_2. Eggs were allowed to stand in oil without agitation, while those in the gas phase were placed on the bottoms of vials and continuously flooded with either N_2 or air. When a liquid medium was used, eggs were covered to a depth of 3 cm. The number of repetitions of each experiment can be derived by dividing the total number of eggs (column 3) by 4.

Medium	Treatment	Eggs (No.)	Hatched (%)	Hatching (minutes)*	Experimental time (hours)
Water	None	24	100	96 ± 111	4
Water	Air	12	16.6†	225 ± 134	8
Water	O_2	12	0.0		3, 4, 10.5
Water	N_2‡	12	100	23 ± 8	0.7
Oil§	None	24	100	33 ± 8	4
Gas	N_2‖	12	100	44 ± 11	1
Gas	Air	24	0.0		48

*Values are means ± 1.0 standard deviation. †Larvae hatched after the aeration system became clogged for about 2 hours. ‡Eggs were placed in O_2 saturated water for 3, 4, and 10.5 hrs (line 3) before transferring to water equilibrated with N_2. §Partially hydrogenated soybean cooking oil. ‖Gases were bubbled through water before passing into the inflow valve of experimental chambers to prevent egg desiccation.

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Table 2. Effects of gas composition and temperature on the hatching of *A. opacum* embryos. In all experiments eggs were placed into vials, covered with water to a depth of 3 cm, and equilibrated with the experimental gases. Gases were passed through separate flow meters at rates that corresponded to the mixture proportions, then mixed in a common chamber before being passed to the inflow valve of vials.

Gas mixture (%)			Temperature (°C)	Eggs (No.)	Hatched (%)	Time (minutes)	
O_2	N_2	CO_2				Hatching*	Experimental
100			23	4	0.0		180
95		5	23	4	0.0		120
90		10	23	4	0.0		120
21	79	0†	23	8	0.0		120
19	76	5	23	4	0.0		120
	100		10	4	100	90 ± 14	111
	100		23	8	100	52 ± 11	65
	100		31	4	100	36 ± 08	45
	95	5	23	8	100	22 ± 06	30
	90	10	23	4	100	28 ± 14	48

*Values (minutes) are the mean ± 1.0 standard deviation. †Air; CO_2 is about 0.03 percent.

twice as fast as those that were not exposed to CO_2 (rows 7, 9, and 10).

In an attempt to understand the accelerating effect of high PCO_2 on hatching, the *p*H of aerated water was determined before and after equilibration with N_2, or a mixture of N_2 (95 percent) and CO_2 (5 percent). The *p*H of aerated water was 7.4, while that of water equilibrated with 100 percent N_2 or with 95 percent N_2 and 5 percent CO_2 was 8.2 and 6.0, respectively. Because hatching involves the action of a degradative enzyme (*7*), it seemed plausible that the effects of CO_2 on hatching times observed in Table 2 were related to *p*H rather than PCO_2. Subsequent experiments in which the *p*H of the hatching medium was modified with phosphate buffers show that hatching was most rapid between *p*H 6 and 7 (Fig. 1). Furthermore, the mean hatching time of embryos placed in phosphate-buffered water equilibrated with 95 percent N_2 and 5 percent CO_2 at *p*H 5 to 7 was not significantly different from that of embryos in phosphate-buffered water equilibrated with 100 percent N_2 at similar *p*H (analysis of variance; $F = 2.19$; d.f. = 5,34; $P > .05$).

In order to determine the range of O_2 pressures over which embryos hatch, the PO_2 in eight vials containing one egg each was decreased in stepwise fashion beginning with aerated water (*p*H 7.4, PO_2 = 150 torr). Embryos were maintained at each PO_2 for 1 hour before they were shifted to lower values (*8*). Hatching did not occur until PO_2 was reduced to 86 torr. At this PO_2, six of eight embryos hatched. The last embryo in this series did not hatch until the PO_2 was below 57 torr.

Our experiments indicate that the hatching process is affected by temperature, *p*H, and PCO_2. However, only lowered PO_2 is essential to the process, and the other factors are ineffective at high PO_2. Within normal environmental ranges, the accelerating effect of increased temperatures on hatching may be the result of several temperature-dependent factors including increased embryonic O_2 demands, reduced environmental O_2 availability, and increased rates of enzymatic activity (*9–12*). Explanations for the rapid hatching of embryos at *p*H 6 to 7 are less obvious. Most developing amphibian embryos are surrounded by two aquatic environments, one within and one outside the egg capsule, and both are normally at or below *p*H 7 (*13*). However, isolated hatching enzymes of amphibians and other organisms have a *p*H optimum for degrading proteins at or near 8 (*10–12, 14, 15*). The accelerated hatching rate of *A. opacum* at *p*H 6 to 7 could imply that this enzyme has an unusual *p*H optimum or is secreted more rapidly at *p*H 6 to 7. During the release of hatching enzymes by fish embryos, the fluid inside the egg capsule may become alkaline (*14*). If the same is true for *A. opacum*, the *p*H effect shown in Fig. 1 may be related more to the rate of enzyme release than to its activity.

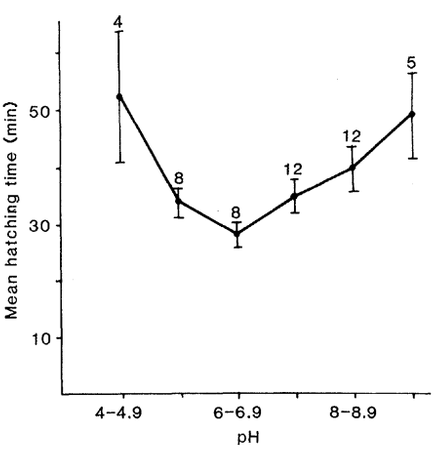

Fig. 1. Effects of *p*H on hatching of *A. opacum* embryos. Eggs were placed into vials at room temperature (22° to 24°C), covered to a depth of 3 cm with 0.1*M* phosphate buffer, and equilibrated with 100 percent N_2. Differences in *p*H before and after each experiment were less than 0.2 *p*H units. Dots represent the means; vertical bars indicate ± 1.0 standard deviation; numbers above the vertical bars indicate sample size.

Our experiments indicate that hypoxia is the hatching trigger for *A. opacum* as well as for other amphibians that we have tested (*16*). Intraspecific and interspecific variations in the hatching stage of amphibians may simply reflect differences in the developmental stages when embryos experience sufficient hypoxia to trigger hatching (*17*). Similar studies with fish embryos have provided either direct (*18, 19*) or indirect (*20*) evidence that the stage of hatching is dependent upon the establishment of hypoxic conditions. Hatching can be delayed by exposing the embryos of some fish species to pure O_2 or by maintaining adequate circulation of aerated water. In addition, hatching can be induced by exposing these fish embryos to 100 percent N_2. Hypoxia also is the apparent trigger for hatching in several invertebrates including mosquitos (*21*) and freshwater copepods (*22*). Other environmental factors such as osmotic pressure (*19, 23*) and light intensity (*24*) apparently can also serve as hatching triggers. The effects of these environmental factors on the O_2 consumption of embryos have not been investigated and it is not known whether hypoxia plays a role in the hatching of these organisms. While there is some question whether hypoxia is a universal trigger for hatching in aquatic organisms (*25*), we believe that it is a far more general phenomenon than that previously thought.

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Transplantation of Leukemic Bone Marrow Treated with Cytotoxic Antileukemic Antibodies and Complement

Abstract. *The ability of antiserum against murine L1210 leukemia to remove residual leukemia cells from murine bone marrow was investigated. Leukemic marrow was treated in vitro with antiserum and complement and used to hematologically reconstitute mice that had been irradiated with doses lethal to bone marrow. Following infusion of treated leukemic marrow, normal marrow returned without evidence of leukemia. More than 90 percent of the animals have survived for 11 months without untoward effects, suggesting that the technique may be of use in the treatment of acute leukemia in humans.*

Transplantation of allogeneic bone marrow is often used to treat leukemic patients whose disease has resisted standard therapy (*1, 2*). In this approach, leukemic patients are given chemotherapy and total body radiation to destroy the bone marrow and then receive an intravenous infusion of marrow from a histocompatible donor. The procedure has met with some success but also has certain limitations (*3*). Many patients lack a histocompatible bone marrow donor, and there is significant morbidity from graft-versus-host disease.

An alternative approach involves the reinfusion, following marrow ablation, of autologous bone marrow harvested during a remission (*4*). This method eliminates the need for histocompatible donors and the problem of graft-versus-host disease. However, preliminary attempts with this approach have been disappointing because leukemia has recurred coincident with or before the return of normal hematopoietic activity (*4*). This is presumably due to a reinfusion of viable leukemia cells present in the marrow obtained during "remission" or to a failure of the conditioning regimen. To avoid the problem of infusing leukemia cells, in vitro elimination of leukemia cells from so-called "remission" bone marrow by using specific antiserum to leukemia has been considered (*5*). This approach is now being attempted in some patients (*6*).

In this study we demonstrate that specific antiserum can eliminate leukemia cells from murine bone marrow in vitro and that long-term disease-free survival is possible following reconstitution with the treated marrow.

Antiserum to murine L1210 leukemia (*7*) was prepared in accordance with a modification of a previously described technique (*8*). New Zealand White rabbits were injected intravenously with 2×10^8 viable L1210 cells on eight separate occasions over a 4-week period. Prior to immunization the cells were sedimented through a Ficoll-Hypaque density gradient to remove contaminating red cells and resuspended in 2 ml of undiluted rabbit antiserum to DBA/2 thymocytes. Blood was collected 1 week after the eighth injection. The serum obtained was adsorbed at 37°C against erythrocytes, thymocytes, and splenocytes from C57B1/6 and DBA/2 mice. A 1:4 dilution of the adsorbed antiserum produced 100 percent lysis of leukemia cells in a complement-dependent in vitro assay of cytotoxicity (*8*).

In the first experiment we determined the dose of L1210 leukemia cells required to kill normal DBA/2 mice. Intraperitoneal injection of as few as ten viable cells killed more than 60 percent of the mice within 21 days (Fig. 1A). This indicated that if ten or more viable leukemic cells remained in the leukemic bone marrow following treatment in vitro, the animals reconstituted with such marrow would succumb to recurrent leukemia.

The next experiment was designed to determine the cytotoxic efficacy of the antiserum and normal rabbit complement. Some 10^5 viable cells were incubated with antiserum, complement, or both for 60 minutes at 22°C and then injected intraperitoneally into healthy DBA/2 mice. No deaths occurred in the mice injected with leukemia cells incubated with antiserum and complement (Fig. 1B). In contrast, 100 percent mortality was observed in animals injected with untreated cells or with cells that had been incubated with antiserum or complement alone.

From these studies it was concluded that the animals were unable to survive injections of leukemia cells coated only with heterologous antiserum. Presumably the host was unable to recognize antibody-coated cells as foreign. However, since all animals survived free of leukemia when injected with the antibody and complement-treated cells, our transplantation experiments were planned to treat leukemic bone marrow in vitro with both these components. These experiments were performed as follows.

Bone marrow was obtained from the femurs of female DBA/2 mice. The marrow to be transplanted into one group of mice was treated with an equal volume of hypotonic buffer containing EDTA, NH_4Cl, and $KHCO_3$. Viable L1210 cells were added to yield a final concentration of 2.5 percent L1210 cells in the marrow preparation. Following sedimentation the supernatant was removed and the leukemic marrow pellet was incubated at 22°C for 30 minutes with undiluted, adsorbed, L1210-specific antiserum. Normal rabbit serum as a source of complement was then added in a concentration of 2.5:1 and incubated with the marrow at 22°C for 60 minutes. The incubation mixture was shaken frequently. The treated marrow was then sedimented,

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