THE EVOLUTION OF THE IMMUNE RESPONSE

III. IMMUNOLOGIC RESPONSES IN THE LAMPREY*

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Over the past several years we have been concerned with definition of the origins of adaptive immunity among the lower vertebrates (1, 2), using the term "adaptive immunity" to refer to capacity to form specific antibody, to develop delayed allergy, to reject homografts of skin or other tissues, to show responsive proliferation of lymphoid cells upon antigenic stimulation, and to demonstrate immunologic memory.

Extensive prior studies (3–9) had established that invertebrates deal with infection by means of non-specific bactericidal substances in their body fluids, phagocytosis, inflammation, and a fibrous walling-off process. Although Phillips *et al.* (10, 11) and Osawa and Yabuubhi (12) have presented evidence of processes in the invertebrates which they interpret as immunologic in nature, incontrovertible evidence of their specificity has not been presented.

Our earlier studies (1, 2, 13, 14) of the California hagfish, perhaps the most primitive of the vertebrates, failed to reveal evidence of gamma globulin formation, clearance of a variety of antigens, antibody synthesis, or follicular organization of lymphoid tissue. By contrast, *Amphibia*, *Reptilia*, and the more recent *Elasmobranchii* (15–22) have gamma globulins, are able to clear antigens and make antibody, and possess organized lymphoid tissue and plasma cells. Further studies showed that representative holosteans (22, 23) and chondrosteans (24–26), and certain teleosts (22, 27) have the full range of immunologic responses. Our studies (24, 25), as well as Downey's early report (28) established that even the ancient holostean and chondrostean fishes have a plasma cell system.

Preliminary studies (2) suggested that detailed analysis of the sea lamprey (*Petromyzon marinus*) might contribute critical evidence to our investigation

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of the phylogenetic development of adaptive immunity. In this paper we have documented these additional observations on adaptive immunologic processes in lampreys and have attempted to relate their immunologic activity to lymphoid tissue development.

It is shown that lampreys possess a small amount of protein in the gamma area, form specific agglutinating antibody to *Brucella*, reject homografts of skin, develop a delayed allergic response, and show responsive proliferation of lymphoid cells after antigenic stimulation.

Materials and Methods

Experimental Animals.—More than 1700 lampreys including 300 ammocetes (non-parasitic larval stage), 300 newly transformed adults, and 1100 mature adults (sexually mature, past the parasitic stage) were used. Ammocetes were obtained from the United States Bureau of Commercial Fisheries, Hammond Bay, Michigan. Newly transformed adults were collected in the Pere Marquette River, Ludington, Michigan, during the fall and spring downstream migrations into Lake Michigan. During the spring upstream spawning migration, mature adults were trapped in live boxes near electric weirs in either the Cedar River, Escanaba, or Ocqueoc River, Hammond Bay, Michigan.

Ammocetes and newly transformed adults were held in our laboratory in sand bottom aquaria with aeration facilities at temperatures of either 4–10° or 20°C. Mature adults were held in cement raceways with circulating lake water at the Hammond Bay Laboratory, United States Bureau of Commercial Fisheries. The water temperature in the raceways during our studies from May 15 to July 31, 1963, ranged from 41° to 79°F.

Mature adults ranged in size from 12 to 24 inches with an average length of 17 inches. The weight of the animals averaged 200 gm. The fully developed ammocetes and newly transformed adult lampreys were approximately 6 inches long and weighed 3 to 4 gm. In addition, representative stages of ammocetes ranging in size from 40 to 140 mm were used for studies of the development of the lymphoid system.

Antigenic Stimulation.—Five antigens were used, prepared with 0.85 per cent NaCl, and administered intramuscularly.

Killed *Brucella abortus* cells (United States Department of Agriculture) were washed 3 times in saline. The dosages were 1×10^9 cells or 1×10^7 cells in 0.5 ml for adult animals and 1×10^9 cells in 0.1 ml for ammocetes and newly transformed lampreys.

Hemocyanin purified by centrifugation from plasma of keyhole limpets supplied by Pacific Biomarine Supply, Venice, California, was given in dosages of either 10 or 1 mg/kg body weight in a volume of 0.5 ml.

Bovine serum albumin (BSA) (Armour Pharmaceutical Company, Kankakee, Illinois), injected in 0.5 ml volume in dosages of either 10 or 1 mg/kg body weight.

Bovine gamma globulin (BGG) (Nutritional Biochemicals Corp., Cleveland, Ohio) in a concentration of 13.75 mg/ml was mixed 1:1 with complete Freund's adjuvant fortified with *Mycobacterium tuberculosis* strain H37RV kindly supplied by Eli Lilly Co., Indianapolis. The adjuvant was prepared with 17 cc bayol (Esso Standard Oil Company, New York), 3 cc arlacel B (kindly furnished by the Atlas Powder Company, Wilmington, Delaware), and 500 mg of *Mycobacterium tuberculosis*. Each animal received 0.2 ml of the mixture containing approximately 10 mg of BGG/kg body weight.

Bacteriophage T_2 (*Escherichia coli B*) as previously described (2) and suspended in saline was injected in dosages of 1×10^{10}, or 1×10^7 particles in 0.5 ml for adult lampreys and in dosages of 1×10^{10}, 1×10^9, 1×10^8, 1×10^7, and 1×10^5 particles in 0.1 ml for ammocetes and newly transformed animals.

Groups of 50 to 60 animals were immunized with each dosage of each of the 5 antigens. Ten to 15 adult lampreys were bled and sacrificed 11, 21, and 32 days after primary stimulation. Surviving adult animals in each group were restimulated with the same antigen 32 days after the initial injection and sacrificed 14 days later.

Additional groups of 50 to 60 adult lampreys were immunized with hemocyanin (10 and 1 mg/kg body weight) and T_2 bacteriophage (1×10^7 and 1×10^{10} particles per animal) and groups of 10 to 15 adult lampreys were bled and sacrificed 30 days after primary stimulation at which time the survivors were restimulated and a group of at least 15 animals bled and sacrificed 11 and 21 days following secondary stimulation.

Blood Collection and Serum Preparation.—Blood samples were collected in sterile falcon plastic test tubes from the conus arteriosus as previously reported (2) and allowed to clot at 4°C overnight. Serum was separated by centrifugation at approximately 2000 RPM in an International model C-S centrifuge. Sera were stored in the refrigerator at 4°C until antigen clearance studies and antibody determinations were made. Blood was collected from ammocetes and newly transformed lampreys in capillary tubes from the severed conus arteriosus. Approximately 0.1 to 0.2 cc of blood was obtained from each animal. The separated sera were pooled prior to analysis.

Tissues for Histologic Examination.—Representative tissues, including tissue from gill regions, protovertebral arch, anterior, mid and posterior gut, liver, and kidney, were taken at each bleeding interval from members of each group. Specimens were preserved in neutral formalin or in absolute ethanol. Sections were stained with hematoxylin and eosin according to the standard techniques, and with methyl green–pyronin according to the method of Opstad (29).

Smears of peripheral blood were prepared by usual techniques, dried rapidly in air, and stained with Wright-Giemsa. Imprints prepared from protovertebral arch tissue were stained with Wright-Giemsa.

Titration of Antisera and Test for Circulating Antigen.—Agglutinins against *Brucella* were measured by a standard bacterial cell agglutination method (30). Antibodies against BGG and hemocyanin were measured by the BDB hemagglutination technique (2). Antibody against BSA was measured by the ammonium sulfate precipitation method of Farr (31) and by the BDB hemagglutination technique. T_2 bacteriophage antibody and clearance were measured by the method reported previously (2).

Sera collected from lampreys immunized with BSA, BGG, and hemocyanin were tested for circulating antigen by incubating lamprey sera with rabbit anti-BSA, BGG, or hemocyanin serum in capillary tubes. Circulating T_2 bacteriophage was measured by counting the number of viable phage particles per milliliter of lamprey serum (2).

Delayed Allergic Response.—Lampreys stimulated with BGG and Freund's complete adjuvant fortified with *Mycobacterium tuberculosis* strain H37RV 25 mg/ml were skin-tested with a 1:10 dilution of old tuberculin (O.T.) (Parke Davis Co., Detroit) 21 and 30 days after primary stimulation. Ten normal animals were similarly tested with O.T. at each time interval.

Skin-Grafting Procedure.—Twenty-three mature adults in good physical condition were selected for skin grafting. The animals were lightly anesthetized with 0.01 per cent 4-styrylpyridine (Eastman Organic Chemicals Inc., Rochester, New York) in lake water (32); 1 x 2 cm full thickness autografts of skin were placed on the lateral side on each animal and identical sized full thickness homografts of skin were exchanged in similar location between pairs of animals. The grafts were secured with 5-0 silk using 3/8 inch circle atraumatic needles. Grafts were inspected every 3 to 4 days and grafted skin and wounds were treated by topical application of 1 per cent malachite green solution at the first signs of fungus infection.

Endotoxin.—Twenty-five small adult lampreys ranging from 33 to 128 gm with an average of 59 gm were used for study of toxicity of Gram-negative bacterial endotoxin. The endotoxin was kindly supplied by Dr. W. W. Spink and was designated lot 453. It had an LD_{50} in 20 gm

Swiss Webster mice of 0.5 mg intraperitoneally. Endotoxin was injected intramuscularly for analysis of systemic toxicity and intradermally for analysis of the skin toxicity.

Electrophoretic Studies.—Lamprey sera were studied by paper electrophoresis at pH 8.6 in veronal buffer (ionic strength, 0.1) at 60 v and a current of 15 ma. Microimmunoelectrophoresis, as described by Scheidegger (33), was used to characterize serum samples further.

RESULTS

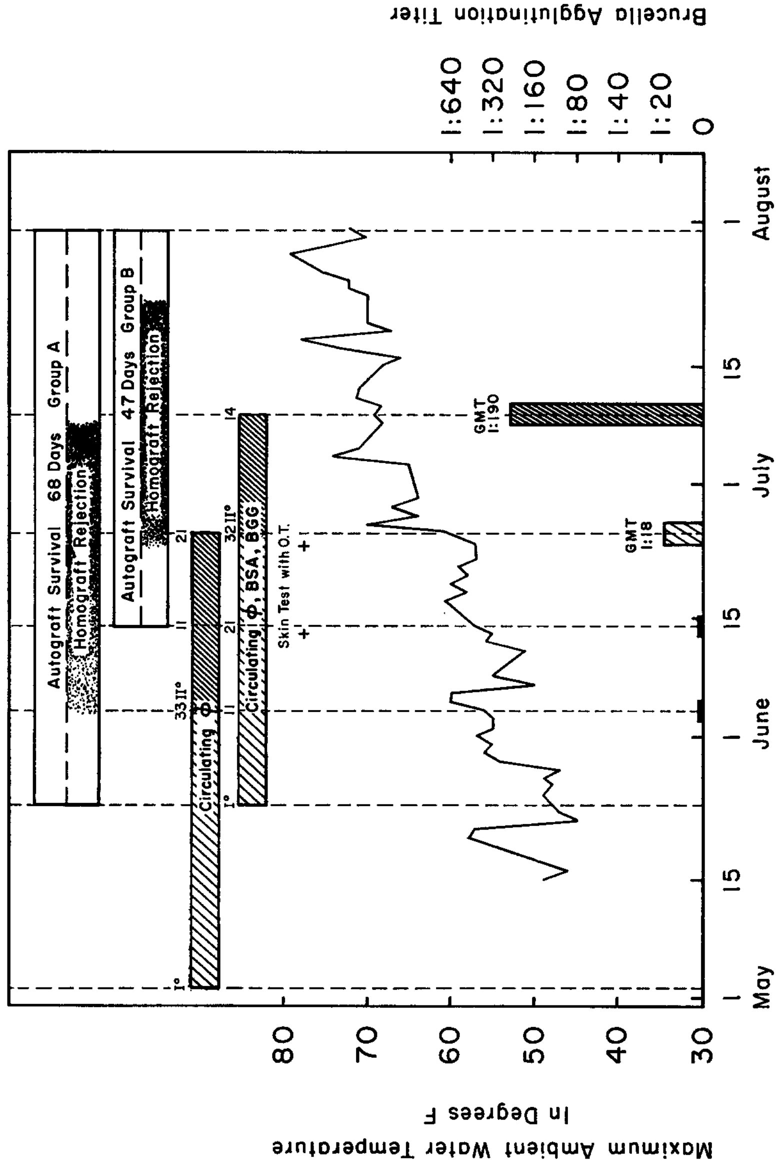
Since temperature is known to play such an important role in antibody production in poikilothermic animals (15, 25, 34), the maximum daily water temperatures of the lake water circulating through the raceways where lampreys were kept for study are recorded in Text-fig. 1. Also indicated on this figure are the times of antigenic stimulation, bleeding, skin testing, and homografting of the experimental animals, as well as the time of appearance of anti-*Brucella* antibody.

Antigen Clearance and Antibody Production to Soluble Protein Antigens.—All of the lampreys immunized with bovine serum albumin (BSA) (10 and 1 mg/kg) had circulating antigen for 32 days after primary stimulation and 14 days after secondary stimulation. No specific antibody to BSA could be found in the sera of these animals by capillary tube precipitation, Farr technique, or BDB hemagglutination techniques. All animals given bovine gamma globulin (BGG) circulated this antigen up to 30 days following its injection in Freund's adjuvant. Adjuvant stimulation resulted in a high mortality rate, and study of BGG clearance beyond 14 days following secondary stimulation was not possible. No antibody was demonstrated against BGG using BDB hemagglutination techniques.

Hemocyanin, on the other hand, was cleared from the circulation, but, in this study, no antibody against this antigen was found by hemagglutinin and precipitin techniques. These observations are summarized in Table I.

Clearance and Antibody Synthesis with Bacteriophage.—Lampreys immunized with 1×10^{10} and 1×10^7 viable T_2 bacteriophage per animal demonstrated circulating viable phage for 32 days after primary stimulation and 32 days following secondary stimulation. These observations are summarized in Table I. In our prior investigations it was shown that T_2 bacteriophage was rarely eliminated from the circulation when administered to lampreys intramuscularly in a dose of 1×10^{10} particles (2).

In this study 8/111 lampreys showed complete disappearance of bacteriophage from the circulation. One of these had been given 1×10^{10} phage particles and 7 had been given 1×10^7 phage particles. Sera from each of these lampreys and all sera from lampreys with less than 100 phage particles per ml were analyzed for capacity to neutralize the phage. Even though phage neutralization is a sensitive means of detecting antibody only one of these "immune" sera neutralized T_2 bacteriophage at a rate significantly faster than that observed to be characteristic of pooled normal lamprey serum. This ani-



TEXT-FIG. 1. Relationship of antigenic stimulation and immunologic responses to ambient water temperature. I⁰, primary stimulation; II⁰ secondary stimulation, GMT, geometric mean titer.

mal neutralized 76 per cent of the bacteriophage added within 6 hours upon incubation at room temperature. By contrast, a veronal buffer control and 10 per cent normal rabbit serum control did not neutralize the phage while a pool of 5 non-immunized lamprey sera neutralized 27 per cent over the same 6 hour period upon incubation at room temperature. None of the other "immune" lamprey sera studied from representatives of animals stimulated with 1×10^7 or 1×10^{10} phage particles neutralized a significantly greater percentage of the phage than did the pool of normal lamprey sera. Ammocetes and newly transformed adults were immunized in groups of 50 with bacteriophage including 1×10^{10}, 1×10^9, 1×10^8, 1×10^7, and 1×10^5 phage particles per

TABLE I

Antigen Clearance and Antibody Production in the Adult Lamprey

Antigen dose	Antigen cleared from serum*	Antibody production	Method
10^{10} T_2 phage/animal	1/49	0/49	Phage neutralization
10^7 T_2 phage/animal	7/62	1/62	Phage neutralization
BSA 10 mg/kg body weight	0/35	0/35	Farr I^{131} Capillary precipitin BDB hemagglutination
BSA 1 mg/kg body weight	0/36	0/36	Farr I^{131} Capillary precipitin BDB hemagglutination
BGG in adjuvant 10 mg/kg body weight	0/9	0/9	BDB hemagglutination
KHL hemocyanin 10 mg/kg body weight	71/71	0/71	BDB hemagglutination
KHL hemocyanin 1 mg/kg body weight	66/66	0/66	BDB hemagglutination

* Viable bacteriophage was detected in circulation by method previously described (2). Circulating BSA, BGG, and hemocyanin were detected by a capillary tube precipitation method using rabbit antisera specific for each antigen (2).

animal. Sera of these animals were studied at intervals over a 3 month period. In every instance failure to completely eliminate bacteriophage from the circulation was observed since greater than 100 viable phage particles per ml of serum were recovered in every pool studied from the 5 groups.

We conclude from these experiments that the lamprey at all stages of its life cycle does not regularly eliminate phage from the circulation and forms little or no neutralizing antibody to the bacteriophage even upon repeated stimulation. Further, even in those few instances when bacteriophage was finally cleared from the blood, no detectable antibody was found.

Antibody Production in Adult Lampreys Immunized with Killed Brucella Cells.—Adult lampreys immunized with 1×10^9 and 1×10^7 killed *Brucella* cells demonstrated agglutinating antibody 32 days after primary stimulation and

14 days after secondary stimulation. The specificity of the antibody was established by cross-absorption studies with killed *Brucella* cells and typhoid-paratyphoid cells. The antibody was completely absorbed by killed *Brucella* cells while the agglutination titer of the serum was not reduced by prior absorption with the typhoid cells. Individual and pooled normal sera did not agglutinate killed *Brucella* cells. These data are presented in Table II. It will be seen from the table that 32 days following primary stimulation with 1×10^7 cells the geometric mean agglutinin titer reached 1:8. Three of 5 animals stimulated with 1×10^7 cells and 7 of 10 stimulated with 1×10^9 cells formed antibody. The geometric mean titer in the group stimulated with 1×10^9 cells was only

TABLE II

Anti-Brucella Antibody in the Adult Lamprey

Days	Stimulation	Dose	Antibody	Geometric mean titer	Range of titer
11	Primary	1×10^7	0/6	0	
		1×10^9	0/6	0	
21	Primary	1×10^7	0/6	0	
		1×10^9	0/6	0	
32	Primary	1×10^7	3/5*	1:8	1:20 1:80
		1×10^9	7/10	1:18	1:20 1:320
14	Secondary	1×10^7	2/17	1:2	1:20
		1×10^9	14/14	1:190	1:40 1:2560

* Numerator indicates number of animals with positive anti-*Brucella* agglutination titers. Denominator indicates total number animals tested.

1:18 at this time and titers, when present, ranged from 1:20 to 1:320. Fourteen days following secondary stimulation, only 2/17 of the animals immunized with 1×10^7 cells showed circulating antibody and each showed a titer of 1:20. By contrast, 14/14 of those secondarily stimulated with 1×10^9 cells showed antibody with titers ranging from 1:40 to 1:2560 with a geometric mean titer of 1:190.

Brucella Antibody in Newly Transformed Adults and Ammocetes.—Groups of 50 ammocetes and newly transformed adult lampreys were immunized with 1×10^9 *Brucella* cells and kept either at room temperature or at 4–10°C for a period of 2 weeks following primary stimulation. Mortality of both stages of the life cycle was high at room temperature. Sera from 5 to 12 animals were pooled for analysis. Unstimulated controls showed no capacity to agglutinate *Brucella* cells even at very low dilution. Agglutinins were present in the serum of immunized lampreys in dilutions of 1:16 and 1:32. Surprisingly, antibody

in this amount seemed to be produced as well at both temperatures. We conclude from these observations that small amounts of antibody are produced in the ammocete and newly transformed adults following primary stimulation with killed *Brucella* cells.

Adjuvant Reaction and Delayed Allergic Response in the Lamprey.—The highest mortality rate among adult lampreys receiving antigenic stimulations occurred in the group given BGG with complete Freund's adjuvant fortified with *Mycobacterium tuberculosis*. Of 42 animals initially injected with BGG and adjuvant, 26 died within 21 days after stimulation. The loss of immunized animals in other groups of 50 to 60 animals was minimal, the greatest losses in any one group being less than 10 per cent over the entire course of the experiment. By the 11th day only 2 animals had died in the adjuvant stimulated group. The remaining animals were heavily infected with superficial fungus which covered 70 to 80 per cent of total body area. The site of adjuvant injection was evident in all animals examined and was characterized by swelling, discoloration, and induration.

On the 21st day after the stimulation surviving lampreys were skin tested with old tuberculin (O.T.) diluted 1:10. Twenty-four hours later 5/7 showed areas of induration and inflammation 3 to 8 mm in diameter. In each of these animals the site of adjuvant injection showed induration and inflammation. Two of 7 had negative skin tests with old tuberculin. Both of these animals also showed no apparent reaction at the site of adjuvant injection. Ten animals not previously stimulated with *Mycobacterium tuberculosis* strain H37RV fortified adjuvant were used as controls and in no instance was a positive skin test present. Indeed, the site of administration of the O.T. could scarcely be detected 24 hours after injection. The two lampreys with negative skin tests and one with positive skin test were saved to be tested 9 days later. By the 30th day after primary stimulation, both animals previously negative to tuberculin testing were now positive. The site of adjuvant injection in the 2 animals also was evident as a swollen indurated area. The animal that had shown a positive skin test 9 days before again showed a positive skin test with O.T. at 30 days.

In most of the animals stimulated with Freund's adjuvant supplemented by *Mycobacterium tuberculosis* the inflammatory reaction was severe enough to produce at the initial injection site necrosis and sloughing of the skin extending down to the myotome layer. The delayed allergic reaction to old tuberculin in the adjuvant-stimulated lamprey is compared to the injection site of 0.1 cc of 1:10 dilution of O.T. in an unstimulated control lamprey in Fig. 1.

Responsive Cellular Proliferation in the Hematopoietic Tissue of the Protovertebral Arch.—The fibrocartilaginous protovertebral arch of the lamprey houses a tissue often referred to as the "fat pad" of this animal. This tissue has been shown by our prior investigations to contain, in addition to fatty tissue, hema-

topoietic elements (23, 25). In the unstimulated lamprey the hematopoietic elements are mostly erythropoietic and myelopoietic and the tissue has a morphology reminiscent of mammalian marrow (Fig. 2). In the lampreys stimulated with Freund's adjuvant fortified with *Mycobacterium tuberculosis* a striking proliferative response occurred in this tissue (Fig. 3). The hematopoietic tissue of the protovertebral arch can be seen in the unstimulated lamprey to be composed largely of erythroid and myeloid elements, and a few phagocytic cells are present (Figs. 4 *a* and 4 *b*). By contrast, following stimulation with antigen in Freund's adjuvant, the tissue develops foci of round cells, identifiable as lymphoid elements, which are illustrated in Figs. 5 *a* and 5 *b*.

TABLE III

Homograft Rejection in the Adult Lamprey

Group	No. of lampreys
Total number lampreys autografted and homografted	23
Dead at 11 days	7/23
Technical failure of one or both grafts	7/16
Technical success of both grafts	9/16
Autograft rejection*	0/9
Homograft rejection‡	9/9

* Autografts remained in place as long as 68 days.

‡ Rejection occurred between 21 and 42 days following placement.

In another study we also observed that following injection of radioiodinated hemocyanin this antigen was concentrated primarily in the gill region and could also be detected in the hematopoietic tissue of the protovertebral arch within 24 to 48 hours.

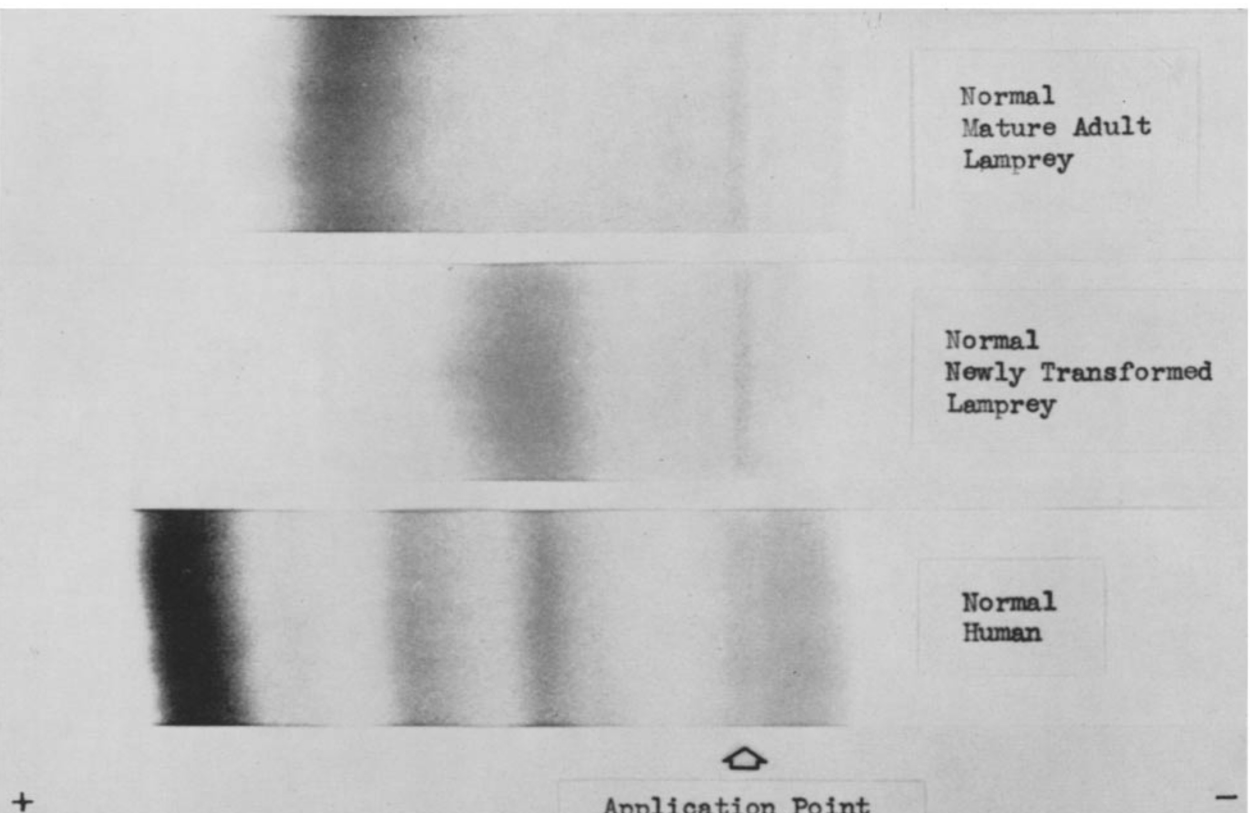
Homograft Rejection in the Adult Lamprey.—Summarized in Table III are observations concerning behavior of homografts and autografts of skin in the lamprey. Twenty-three animals received both autografts and homografts of skin. Of these, 7 had died by the 11th day after placement of grafts. In 7 of the remaining animals either the autograft or the homograft was technically unsatisfactory and did not heal in place. In the 9 others both autograft and homograft were technically successful, healed in place, and were viable 11 days after placement. Of the 9 autografts, all remained in place until the death of the animal 47 to 68 days following transplantation. By contrast, complete rejection of each of the 9 homografts occurred between 21 and 42 days following placement. Beginning signs of rejection were sometimes apparent as early as 11 days. Of those animals in which either the autograft or homograft was successful, the behavior of the grafts, although not strictly controlled by the presence of a technically successful paired graft, was similar

to that observed in the controlled specimens. The results are summarized on Table III. Fig. 6 compares an autograft and homograft in the same animal at 11 and 47 days. It may be seen in the figure that the autograft remains healthy and maintains integrity even of the skin pigment throughout, while early signs of homograft rejection are apparent 11 days following transplantation. The homograft has been sloughed and is replaced by scar tissue 47 days after placement of the graft.

Effect of Escherichia coli Endotoxin.—Lampreys failed to respond to *E. coli* endotoxin when the latter was injected intramuscularly in dosages ranging from 0.168 to 168 mg/kg body weight. In these studies 25 animals were used. Animals in each group were injected intramuscularly with 0.168, 1.68, 16.8, and 168 mg/kg. The animals were observed 15 minutes, and 1, 24, and 48 hours following injection. Their swimming behavior, gill action, capacity to attach with their oral sucker, appearance of fins, and general appearance were observed. At no time did any of these animals show evidence of ill effects of this endotoxin. Studies to evaluate capacity of the *E. coli* endotoxin to injure the skin revealed no reaction over a 48 hour period following intradermal injections of 1.68 and 16.8 mg/kg in 0.1 ml of saline.

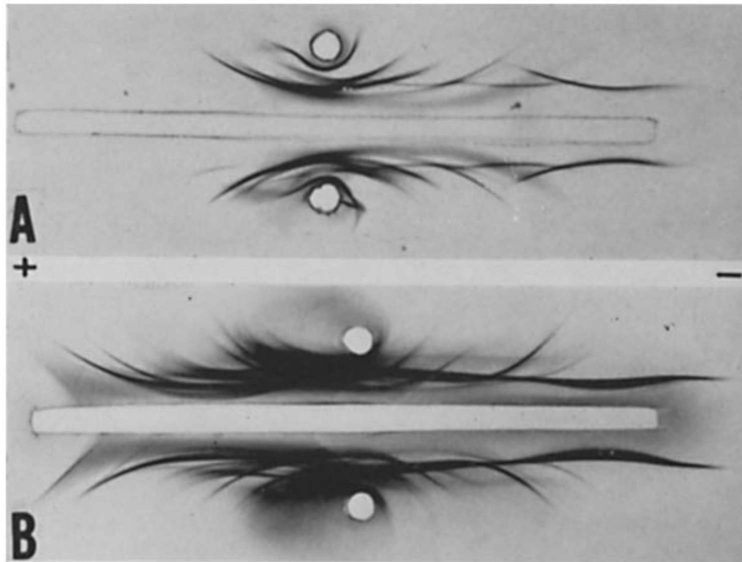
The Lymphoid Tissue of the Lamprey.—Detailed observations and illustrations of the development and structure of the lymphoid tissue in the lamprey are presented elsewhere (25). In the light of the immune responses presented here, however, it is of importance to summarize briefly these observations. Extensive studies of smears and imprints of the peripheral blood, gill region, protovertebral arch–hematopoietic tissue, and hematopoietic tissue in the anterior gut failed to reveal cells of the plasma cell series even after antigenic stimulation. In the peripheral blood one could identify numerous monocytic elements, and a family of large, medium, and small lymphocytes. Focal accumulations of lymphoid cells in all stages of development could be identified in the primitive spleen located in an infolding of the anterior gut. This tissue is primarily reticular, erythropoietic, and myelopoietic tissue, but toward the periphery of the hematopoietic tissue in this infolding, one may identify clusters of small round cells which we consider to be primitive lymphoid foci (Figs. 7 *a* to 7 *c*). As was originally observed by Salkind (35) we, too, located small accumulations of lymphoid cells in groups of 5 to 20 cells scattered in the epithelium of the peripharyngeal gutter from the second to the fifth gill arches. We considered this lymphoid tissue to represent the most primitive thymus (Fig. 8). No truly separate organ can be identified as a thymus or a spleen in the lamprey. Finally, lymphoid foci appear in the protovertebral arch only following adjuvant stimulation as was mentioned above (Figs. 5 *a* and 5 *b*).

Nature of the Serum Proteins in the Lamprey.—Electrophoretic and immunoelectrophoretic studies revealed clear evidence of a small amount of gamma globulin in normal adult lamprey sera (Text-figs. 2 and 3). Studies of the physi-



TEXT-FIG. 2. Paper electrophoresis patterns on adult and newly transformed lamprey sera. A normal human serum pattern is included for comparison. Note the absence of an albumin component and the presence of small amounts of protein with the mobility of gamma globulin on these patterns. The adult lamprey at this stage of its life cycle is non-parasitic.

cal chemistry of the serum proteins in these fishes and the quality of antibodies produced were made under direction of Dr. B. Pollara as part of an extensive study of protein and immunochemistry in primitive fishes and will be reported elsewhere (36, 37). Ultracentrifugal analysis of lamprey sera showed proteins having sedimentation coefficients of approximately 17S, 8S, 7S, and 3S, as measured in whole serum and extrapolated to infinite dilution. The *Brucella* antibodies produced in these investigations were studied by sephadex G-200 column fractionation and sucrose density gradient techniques



TEXT-FIG. 3. Comparison of immunoelectrophoretic patterns from adult lamprey serum (*A*) and normal human serum (*B*). Note the absence of albumin and presence of proteins in the gamma region. Specific identification of these proteins as immunoglobulins awaits further study.

and shown to be of large molecular size but apparently intermediate between the 17S and 8S components (36). These antibodies were inactivated by 2-mercaptoethanol.

DISCUSSION

We submit that in the lamprey we have comprehensively studied the most primitive adaptive immune response thus far defined. The lamprey met, with the possible exception of the capacity for immunologic memory, our criteria for adaptive immune response. Although these animals were poor antibody producers, they were able to form specific antibody to *Brucella* antigen following both primary and secondary stimulation. They showed responses cer-

tainly definable as delayed allergic responses, were able to reject skin homografts while accepting autografts and also showed responsive mononuclear cellular proliferation upon intensive antigen-adjuvant stimulation. Thus, these animals meet all the criteria we set down to define adaptive immune responses with the possible exception of immunologic memory. However, a firm conclusion concerning immunologic memory must await further analysis, even though response to the secondary stimulation with *Brucella* showed increased titers after secondary stimulation. The first appearance of antibody occurred when the temperature of the raceway water reached 60°F and we could not be certain that the rise in titer observed was attributable to the second injection of *Brucella* antigen and not to the rise in ambient water temperature. In this regard it is of interest that newly transformed adults and ammocetes formed small amounts of antibody when kept at either 4–10° or 20°C.

As adaptive immunity did not exist among the invertebrates, and is indeed lacking in the lowest known vertebrate, the California hagfish, but appears in a complex form in the lamprey, it might be postulated that the evolution of this process occurred not in a series of developments but in what might be considered, in qualitative terms, a single step. To be sure the process of adaptive immunity appears most feeble by the standards of mammalian responses; the amounts of gamma globulin are very small, the type of antibody protein peculiar and the antibody production to most of the antigens used was not observed. As a matter of fact, the lampreys did not even clear some of the serum protein antigens and bacteriophage from the circulation over a prolonged period of observation. Nonetheless, the lamprey showed the capacity to form specific circulating antibody, reject skin homografts, develop delayed allergic responses, and show responsive proliferation of mononuclear cells. These responsive capacities were coordinate in their appearance with the first phylogenetic appearance of a family of lymphoid cells in the peripheral blood, a primitive thymus, and a primitive spleen with small lymphoid foci. On simple morphologic grounds alone, we lacked evidence to support or reject the hypothesis that the lymphoid cells of the spleen, blood, and protovertebral arch are derived from this primitive thymus. We had hoped at this time that phylogenetic analysis might reveal a true dissociation of cellular and humoral immune responses; however, thus far, neither phylogenetic nor ontogenetic studies have provided clear evidence of dissociation. Perhaps, as would be surprising to some (38, 39), but not to others (40, 41), these different immune responses are part of the same process and are not dissociable except in quantitative terms or at the level of ultimate expression. Further studies of cyclostomes including *Myxine glutinosa* and other lampreys might help to clarify this issue. It is also quite possible that a stepwise development did occur, but that its traces are lost in the extinct progenitors of the lower fishes; *e.g.*, the ostracoderms of the Silurian and Devonian periods or the placoderms of the Paleozoic period. It

seems clear from our studies that immunologic responses can occur in the absence of morphologically recognizable plasma cells. The production of circulating antibody remains feeble and it seems most likely that further analysis of the immune responses in other primitive fishes will provide evidence of the evolutionary step or steps leading ultimately to the capacity for the more vigorous antibody responses against most antigens as observed in higher forms; *e.g.*, birds and mammals. In a subsequent report, we will outline the specific immunologic responses observed in the more primitive sharks and rays, as well as the responses of the holostean, chondrostean, and teleost fishes in an effort to define these steps (26).

As we have stated in previous papers, one of the critical questions concerning the development of immunity is why, not how, this came into being. Studies of the immune response do not provide the answer to this question, but they reveal that the earliest appearance must have been a feeble process capable of more vigorous address to cells than to exogenous agents. Such a process, as we and others have suggested before (1, 2, 7, 42) could well have been a means of control of somatic mutation when the latter was associated with appearance of new antigens, and perhaps not a process primarily useful in its initial form as a protection from exogenous infection. Subsequent development of this system only incidental to its initial purpose may have been reflected in greater ability to resist exogenous infection.

SUMMARY

1. Studies of the immune response have been carried out in more than 1700 lampreys representing three stages in the life cycle of these animals.

2. Lampreys used in this study were unable to clear certain soluble protein antigens and bacteriophage and were unable to make antibodies to these antigens. Hemocyanin was cleared from the circulation.

3. The immune responses demonstrated in lampreys include the production of specific antibody to killed *Brucella* cells, the rejection of skin homografts, and the development of a delayed allergic response to old tuberculin.

4. A responsive proliferation of lymphoid cells occurred in the protovertebral arch following antigen-adjuvant stimulation.

5. Electrophoretic and immunoelectrophoretic analysis of lamprey serum revealed gamma globulin. Ultracentrifugal analysis of serum revealed proteins with sedimentation coefficients of 17S, 8S, 7S, and 3S.

6. The antibodies thus far observed in the lamprey are of relatively high molecular weight and destroyed by 2-mercaptoethanol.

7. In the lamprey it would appear that there is reflected the coordinate evolution of a primitive thymus, primitive spleen containing lymphoid foci, a family of lymphocytes in the peripheral blood and capacity for gamma globulin synthesis and expression of adaptive immunity.

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EXPLANATION OF PLATES

PLATE 128

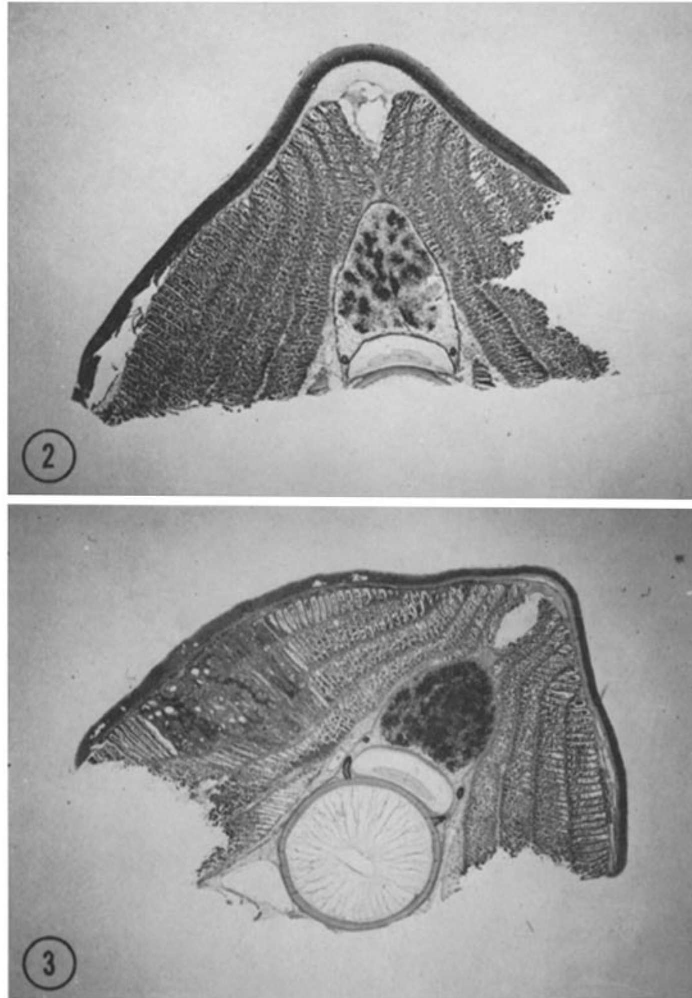
FIG. 1. Delayed allergic response in adult lamprey immunized with complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* organisms and challenged with old tuberculin 21 days after the sensitizing injection. The site of skin testing in an unimmunized lamprey is marked by the arrow.

(Finstad and Good: Evolution of immune response. III)

PLATE 129

FIG. 2. Cross-section of an unimmunized lamprey to illustrate the location and cell density of the hematopoietic tissue in fibrocartilaginous protovertebral arch.

FIG. 3. Cross-section of a lamprey sacrificed 21 days following injection of antigen in Freund's adjuvant. Note the increase in cell density in the hematopoietic tissue of the protovertebral arch region. Note also the tissue destruction at the site of intramuscular adjuvant injection.

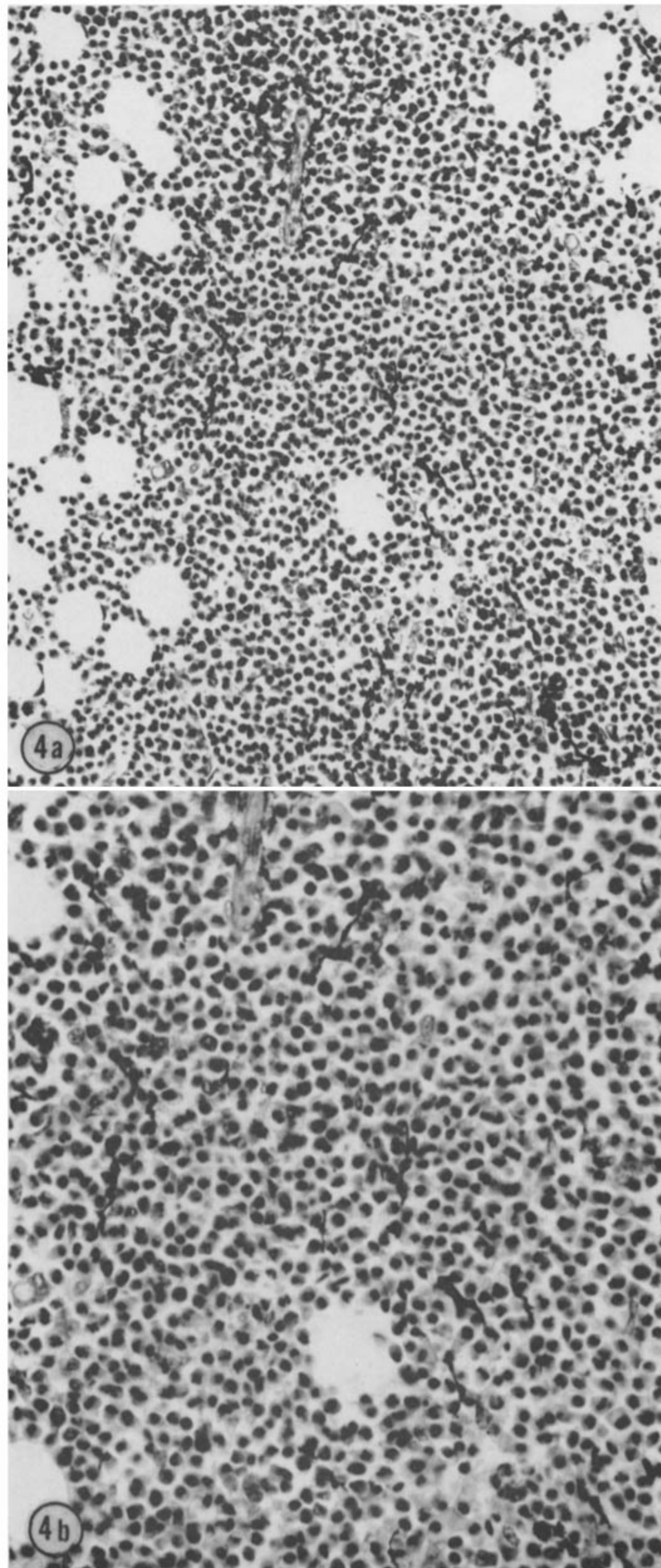


(Finstad and Good: Evolution of immune response. III)

PLATE 130

FIG. 4 *a*. Hematopoietic tissue of the protovertebral arch in unimmunized lamprey. Note the similarity to mammalian bone marrow. Erythropoiesis and myelopoiesis occur here; phagocytosis is also evident. Hematoxylin and eosin. × 250.

FIG. 4 *b*. Higher magnification of hematopoietic tissue of the protovertebral arch in an unimmunized lamprey. Hematoxylin and eosin. × 400.

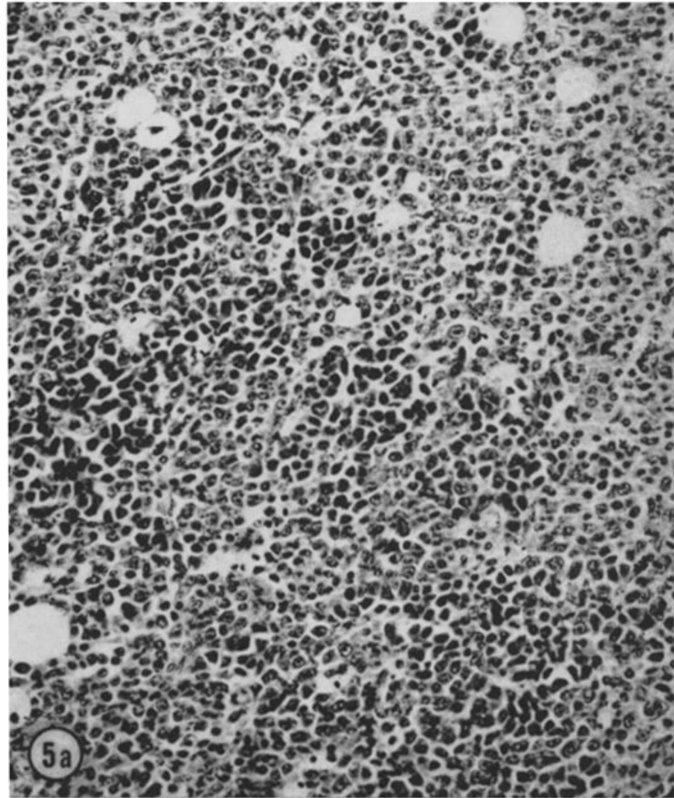


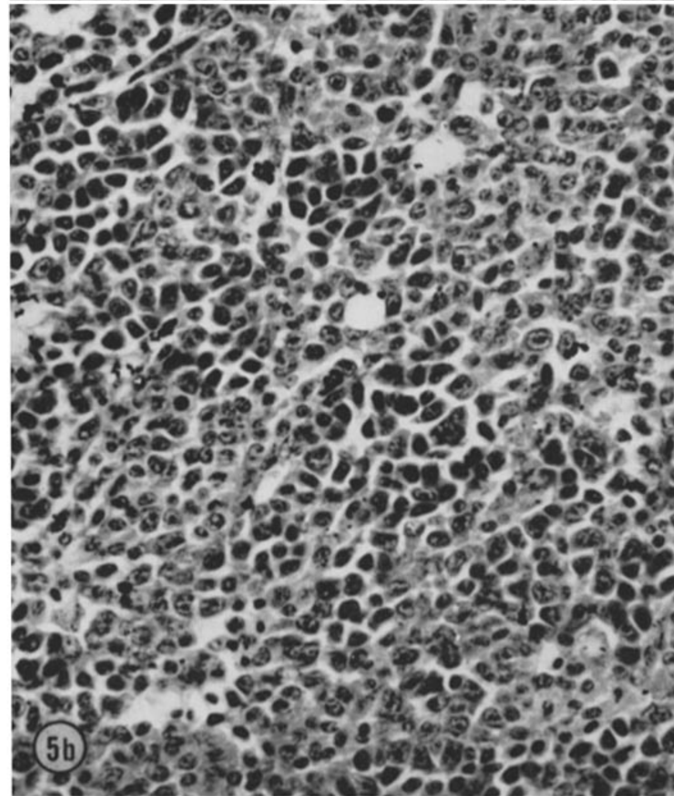
(Finstad and Good: Evolution of immune response. III)

PLATE 131

FIG. 5 *a*. Section of hematopoietic tissue of the protovertebral arch 21 days following injection of antigen in Freund's adjuvant. Note the foci of proliferating lymphoid cells. Hematoxylin and eosin. × 250.

FIG. 5 *b*. Higher magnification of hematopoietic tissue of the protovertebral arch shown in Fig. 5 *a*. Note the proliferating lymphoid cells. Hematoxylin and eosin. × 400.





(Finstad and Good: Evolution of immune response. III)

PLATE 132

FIG. 6. Autograft and homograft on the same lamprey 11 and 47 days after placement of graft. Homograft shows beginning signs of rejection as early as 11 days following transplantation. Homograft has been completely rejected and has been replaced by scar tissue by 47 days. The autograft looks like normal skin at this time.

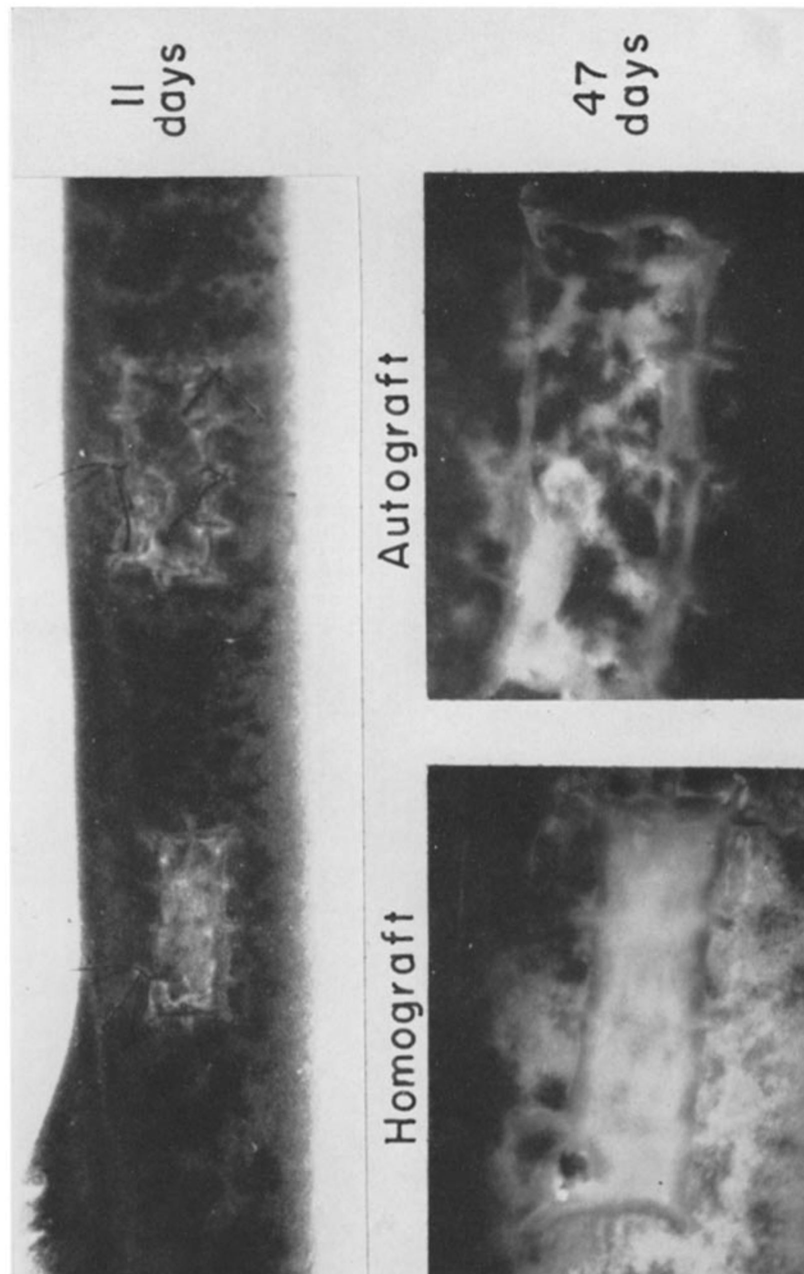


Fig. 6

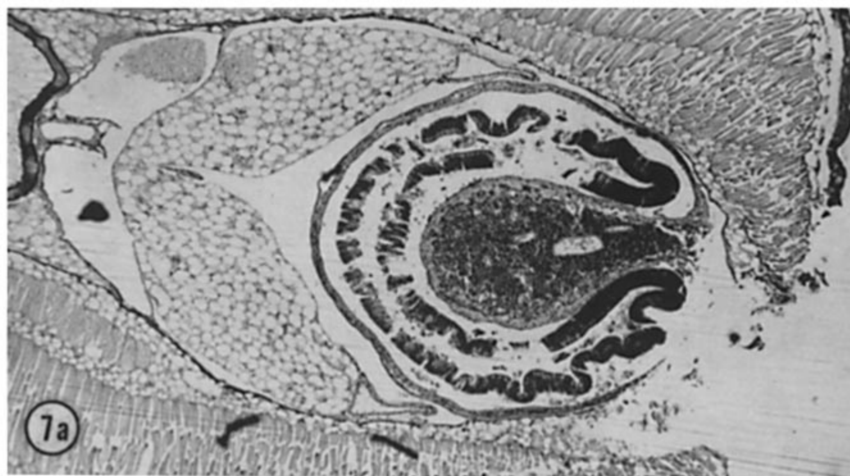
(Finstad and Good: Evolution of immune response. III)

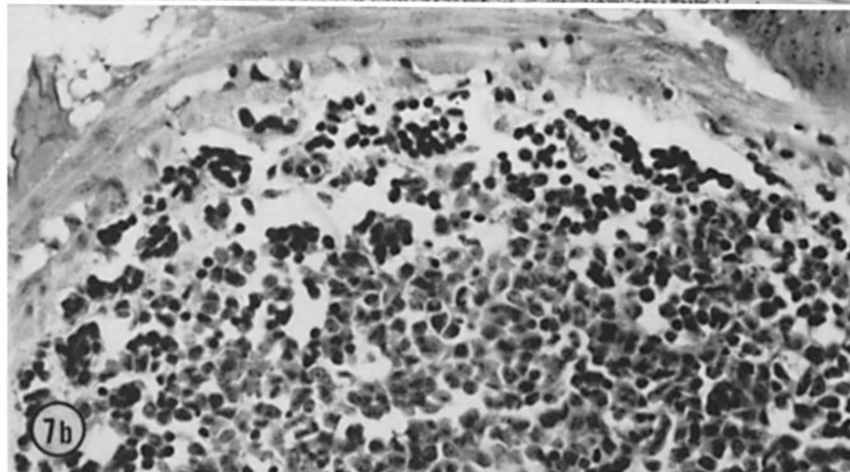
PLATE 133

FIG. 7 *a*. Cross-section of larval lamprey (100 mm) to illustrate location of the primitive spleen in the region of the anterior gut. Hematoxylin and eosin. $\times$ 40.

FIG. 7 *b*. Higher magnification of the primitive spleen to illustrate the focal accumulation of lymphoid cells. The "spleen" is comprised largely of reticular cells. Myelopoiesis and erythropoiesis occur here. Hematoxylin and eosin. $\times$ 400.

FIG. 7 *c*. Higher magnification of the peripheral region of "spleen" to illustrate lymphoid foci. Hematoxylin and eosin. $\times$ 800.


7a


7b

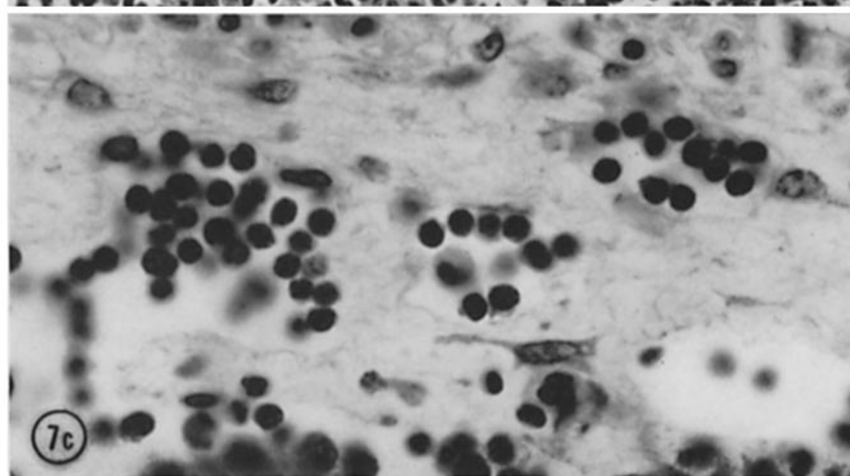
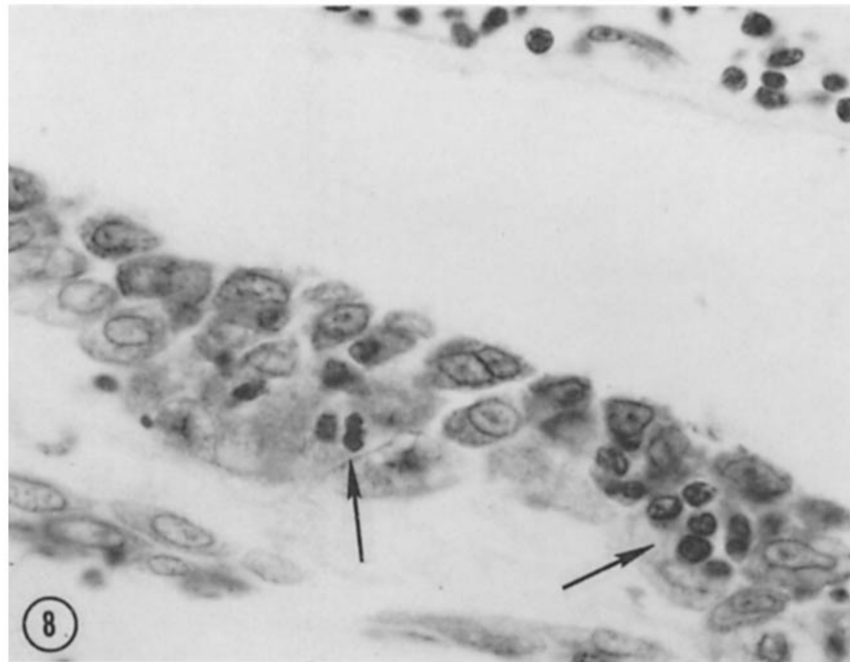

7c

Plate 134

Fig. 8. Lymphoepithelial thymus of larval lamprey (100 mm). Lymphoid cells occur in accumulations of five to twenty cells (arrows) and appear to arise directly from the epithelium. Methyl green–pyronin. × 800.



(Finstad and Good: Evolution of immune response. III)