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Phylogeny of hypersensitivity. I.
Anaphylactic responsiveness of the frog,
Rana pipiens

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Rana pipiens immunized with Salmonella typhosa vaccine developed (anaphylactic) shock reactions on challenge with corresponding soluble bacterial antigen (SBA) but not with washed organisms. Hemagglutinating serum antibody to lipopolysaccharide (LPS) and anti-H agglutinins were demonstrated in responsive frogs. Shock reactions were not influenced by hibernation at 4° C. or by total aqueous immersion. Less uniform results were produced by challenging injections of bovine gamma globulin in 6 of 36 frogs that developed corresponding 2-mercaptoethanol (2-ME)-sensitive hemagglutinating antibodies and 2-ME-resistant precipitins following effective immunization with bovine gamma globulin (BGG) added to typhoid vaccine. Rabbit antiserum to S. typhosa containing anti-O and anti-H agglutinins, 2-ME-sensitive hemagglutinating antibody to LPS, and 2-ME-resistant precipitins to SBA passively transferred anaphylactic responsiveness to the frog, but this could not be effected with rabbit antiserum to BGG. Identification of a shock organ or pathologic changes could not be demonstrated in affected animals. The frog, an ectothermic amphibian representing a transitional position on the phylogenetic scale in production of specific immunoglobulin classes, may develop hypersensitivity manifestations under delineated conditions of exposure and challenge to a microbial antigen.

Study of cellular mechanisms in allergic reactions demonstrates prominent roles for specific leukocyte types. Eosinophils appear to be involved by their response to antigen-antibody complex formation,¹⁻³ phagocytosis of these complexes⁴⁻⁶ and large protein molecular aggregates,⁷ and chemotactic attraction to mononuclear cells sensitized to antigen.⁸ Antigen-antibody complexes are among the physical and chemical agents responsible for disrupting the

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granules of basophils⁹ which in turn may release their content of histamine¹⁰ and heparin¹¹ mediators associated with allergic inflammation.

Two related but divergent species characteristics attracted our interest to the frog. While large numbers of eosinophil and basophil leukocyte types are demonstrable in this animal,^{12, 13} their inability to develop anaphylactic sensitization has been reported.^{14, 15} Yet we have observed and documented susceptibility of the frog to histamine effects.¹³ We were, therefore, especially interested to note a symptom pattern identical to the manifestations of histamine shock¹³ in *Rana pipiens* during courses of immunization with a bacterial vaccine and accordingly have examined this matter in greater detail here.

Observations on hypersensitivity in the frog and the conditions of its occurrence are relevant to the study of evolution of the immune response, a subject that has been extensively reviewed.^{16, 17} The production by amphibians of 19S (IgM class) antibody of the primary response type and sensitive to 2-mercaptoethanol (2-ME)^{16, 18-20} indicates the primitive position of amphibia in the phylogenetic development of immunoresponsiveness. However, under specific conditions of induced antigenic stimulation, the appearance of serum γ G antibodies in frogs and toads has been reported,²¹⁻²⁴ and there is some evidence to suggest that the γ G immunoglobulins emerged at some point in evolution between the lower fishes (elasmobranchs) and the anuran amphibians.²¹ Since the mediation of systemic anaphylaxis has been identified with γ_1 serum antibody in more advanced mammals,²⁵ the possibility of the frog occupying a corresponding transitional position in the phylogenetic evolution of hypersensitivity phenomena seemed worthy of investigation.

MATERIALS AND METHODS

Experimental animals

Rana pipiens of both sexes and weighing 80 to 90 grams were obtained from Lemberger Co., Oshkosh, Wis. They were housed in a porcelain tank with provision for elective bathing in Holtfreter's solution (balanced salt solution containing NaCl 350 mg., KCl 0.5 mg., and CaCl₂ 0.01 mg. per liter), held at 21° C., and fed with mealworms. Injections of all antigens and antisera were given into the cranial lymph sac. Because of temperature influences on antibody production²⁶⁻³⁰ in amphibian models, immunizations and blood samplings were carried out at 21° C. except in studies involving hibernation effects.

Antigens

Salmonella typhosa vaccine was supplied by Eli Lilly Co., Indianapolis, Ind., and was employed at a concentration of 10⁶ killed organisms per milliliter. *S. typhosa* soluble bacterial antigen (SBA) was prepared by ultrasonic treatment of vaccine organisms and employed in a concentration of 100 μ g per milliliter. This represented the soluble fraction recovered from 10⁵ bacterial cells. Bovine gamma globulin (BGG) (Armour Laboratories, Kankakee, Ill.), when used for rabbit immunization, was emulsified in complete Freund's adjuvant and given by subcutaneous and footpad injections.

Serum antibody studies

Agglutination titers were determined by microscopic slide tests (Widal reaction) utilizing both O and H *S. typhosa* bacterial antigens (Difco Laboratories, Detroit, Mich.). A micro-modification of the hemagglutination technique³¹ was performed with the use of: (1) BGG-coated tanned sheep erythrocytes and (2) 50 mg. *S. typhosa* lipopolysaccharide (LPS) (Difco) adsorbed to 1 ml. of 2 per cent sheep red blood cells.³² A modification of the BGG-latex

agglutination method³³ with polystyrene latex particles (Dow Chemical Co., Midland, Mich.) was used to corroborate these results. Precipitins to BGG and to *S. typhosa* SBA were evaluated by interfacial layering of antigen dilutions (BGG 10 to 50 mg., SBA 0.1 mg., and LPS 10 mg. per milliliter) on serum (ring test) and by single-dimension double diffusion of reactants in 1.7 mm. diameter glass tubes filled with agar according to the technique of Preer.³⁴ Duplication of antibody studies was carried out with serum samples 2 hours after treatment with equal parts of 0.2M 2-ME.

Passive cutaneous anaphylaxis (PCA)

PCA in albino rabbits and guinea pigs was performed as described by Revoltella and Ovary.³⁵ Following intracutaneous transfer of rabbit antisera dilutions, 50 μ g of SBA or 1 mg. of BGG in 0.5 per cent Evans blue dye was given intravenously after latent periods of 24 and 72 hours in rabbits and 4 hours in guinea pigs. Reactions were read 30 minutes after the challenging injections. Endotoxin effects from the intravenous injection of LPS precluded its use in critical doses of challenging antigen for PCA tests.

Blood cell identification and histologic examination

Blood samples were taken from a cutaneous abdominal vein prior to and 30 minutes after all challenging injections of antigen. At the end of each experimental period, frogs were killed by cervical dislocation, peritoneal fluid was sampled, a blood sample was taken by cardiac puncture, and specimens of internal organs were obtained. Smear preparations were stained with May-Gruenwald-Giemsa solution. Peritoneal fluid was also examined for mast cells by staining with alcoholic toluidine blue. Tissue specimens were fixed in alcoholic formalin and sections were stained with: (1) a 1 per cent alcoholic solution of toluidine blue and (2) hematoxylin and eosin.

Experimental series

Frogs were divided into groups of six for the experimental procedures described below. For each procedure, one group was bled by cardiac puncture for study of serum antibody and a second group of six animals was used in challenge experiments.

Active sensitization

Typhoid vaccine, 0.2 ml., was given at 3 to 4 day intervals for 6 to 9 injections. Five to seven days after the first appearance of symptoms of sensitization, a challenging injection of 0.5 ml. of SBA was given. Frogs that either reacted with symptoms of shock in response to an initial injection of vaccine or failed to demonstrate this manifestation after 10 sequential injections were studied for serum antibody and excluded from immunization-challenge experiments. Anaphylactic sensitization was studied under three different sets of environmental conditions.

1. Immunization and challenge procedures were carried out at 21° C. room temperature as described for standard housing conditions.

2. After immunization, frogs were transferred to an aerated tank containing Holtfreter's solution at 21° C. and subsequently challenged while held completely immersed.

3. After the initial vaccine injections, frogs were placed in styrofoam containers lined with sphagnum moss saturated with Holtfreter's solution, gradually acclimated to refrigerated room conditions and maintained in hibernation during the course of subsequent immunizing and challenging injections.

One group received injections of 5 mg. of BGG added to 0.2 ml. of typhoid vaccine, and others BGG alone. Subsequent challenging injections were made with 1 mg. of BGG.

Passive sensitization

Rabbit antisera, 0.5 ml., diluted 1:10 was given to normal frogs, followed 24 hours later by an injection of corresponding antigen in the same dosage as described above for the active sensitization series. Antibody determinations for the rabbit antisera used are given in Table I.

In control experiments frogs were injected with 0.7 per cent (physiologic) saline, *S. typhosa* SBA, or BGG alone subsequent to injections of normal rabbit serum according to procedures described for passive sensitization experiments.

RESULTS

Sixty frogs (85 per cent) responded to injections of typhoid vaccine with the production of 2-ME-sensitive serum antibodies. These were detectable by both bacterial agglutination and passive hemagglutination of *S. typhosa* lipopolysaccharide-sensitized cells (Table I). In preliminary study, shock-like symptoms were noted in some frogs on the sixth to the ninth injection of *S. typhosa* vaccine. When these frogs were reinjected with equivalent amounts of either washed and resuspended organisms from vaccine or the supernatant portion after centrifugation, recurrences of reactions were demonstrated only with the latter material.

Ten of 70 frogs neither responded to challenging injections of SBA nor produced agglutinating antibody following initial immunization and a repeated 12 week course. All 40 frogs received in 3 sequential lots from the supplier during one 4 month summer-fall period manifested shock symptoms on receiving initial injections of vaccine. Members of these groups were found to have pre-existing levels of LPS hemagglutinating antibody to *S. typhosa*.

With injections of BGG alone given in different trial dosages and immunization schedules, corresponding serum antibody could not be detected. However,

TABLE I. Serum antibody to *S. typhosa* and to BGG raised in actively immunized frogs, and (*Rana pipiens*)

Group No.	Species	Immunization		Agglutinins	
		<i>S. typhosa</i> vaccine	BGG	O	H
	Frog anaphylactically				
1	responsive	X		2	8-128
2	unresponsive	X		0	0
3	Frog		X		
4	Frog	X	X		
5	Rabbit	X		512	512
6	Rabbit		X		

Groups of 6 frogs each.

*Figures are reciprocals of titers. *S. typhosa*: LPS = lipopolysaccharide; SBA = soluble bacterial

†Sensitivity to treatment of serum with 2-ME: figure in parentheses beneath first figure given
‡0 and + = Results of immunodiffusion tests by Preer³⁴ technique with antigen concentrations
ber of serum pools demonstrating this finding. Precipitins were resistant to 2-ME treatment.

§LA = corresponding titers (reciprocals) by latex agglutination technique.

with the addition of BGG to typhoid vaccine, signs of sensitization reaction were noted and/or serum antibody to BGG was demonstrated after 10 to 15 injections.

Results of anaphylactic challenge and findings related to the character of serum antibody for each of the seven experimental groups are summarized in Table II. Signs of shock, when present, appeared immediately after injection of typhoid SBA. In those animals demonstrating the greatest degree of involvement (3+), flaccid paralysis and apparent loss of response to stimulation lasted for at least a 45 minute period of observation. Those less affected (graded 1+, 2+) developed varying degrees of loss of normal posture and equilibrium and inability to retract from normally disturbing stimuli for periods of involvement lasting 15 to 20 minutes. In these a gradual return of apparent perception and function was noted within the following 20 to 30 minutes. Fatal reaction occurring within 45 minutes was noted in only 2 instances, and these occurred in the series of 6 frogs developing shock while immersed. There were no nonspecific shock reaction effects observed to typhoid SBA at a critical dosage of 50 μ g.

Only hyperinflation of the lungs characteristic of the frog's defense response to nonspecific manipulation was found in anaphylactically responsive animals. No abnormalities of the pulmonary parenchyma were seen on microscopic study. Since procedures for direct counting of total circulating leukocytes and eosinophils in slide chambers are not applicable in the presence of nucleated erythrocytes, only differential cell study was feasible. Eosinophils up to 30 per cent and basophils to 19 per cent were demonstrated, but changes in the percentage

the antibody content of rabbit antisera utilized in passive sensitization experiments in frogs

Serum antibody*						
Hemagglutininst		Precipitins‡			PCA	
		S. typhosa		BGG		
		LPS	BGG			
LPS	BGG	SBA	LPS	BGG	SBA	BGG
5		0	0		0	
0		0	0		0	
	0			0		0
	20 (LA10)§			0 (1)		
	5,120 (LA80)			0 (1)		
	5,120 (LA30)			+ (3)		0
4,096 (8)		+	+		0	
	5,120 (1,280)		1 mg.	AbN		+

antigen (see text); PCA = tests for passive cutaneous anaphylaxis in guinea pigs.

† indicates reduction after 2-ME; where not given, titers were reduced to 0.

‡ of SBA and LPS to 10 mg. and BGG 10 to 40 mg. per milliliter. Figures in parentheses are num-

TABLE II. Anaphylactic reactions in frogs (*Rana pipiens*) following active and passive sensitization procedures

Procedure*		Immune state (Group No.)†	Environmental condition	Anaphylactic response‡
Sensitization	Challenge			
Active				
<i>S. typhosa</i> vaccine	<i>S. typhosa</i> SBA	1	21° C.	+++ (3) ++ (3)
vaccine	SBA	1	Immersion at 21° C.	+++ (6)
vaccine	SBA	1	Hibernation at 4° C.	+++ (5) ++ (1)
vaccine	SBA	2	21° C.	0 (6)
BGG	BGG	3	21° C.	0 (6)
vaccine + BGG	BGG	4	21° C.	+++ (2)§ + (4)§
Passive				
Rabbit Ab to <i>S. typhosa</i>	<i>S. typhosa</i> SBA	5	21° C.	+++ (2) ++ (4)
Rabbit Ab to BGG	BGG	6	21° C.	0 (6)

*BGG = bovine gamma globulin; SBA = soluble bacterial antigen prepared by ultrasonic disintegration of *S. typhosa* vaccine organisms; Ab = antiserum used in passive sensitization experiments.

†Groups of 6 frogs each. The numbers in this column refer to corresponding group numbers in Table I giving the results of serum antibody determinations in actively immunized frogs and the properties of rabbit antisera used in the passive sensitization experiments.

‡+++, Complete shock state and paralysis for 30 to 45 minutes; ++, recovery from shock within 10 to 15 minutes or milder symptoms of loss of balance or partial paralysis for 30 to 45 minutes; +, transient mild symptoms (see text). Figures in parentheses indicate number of animals showing this reaction.

§Shock reactions were demonstrated in only 6 of 36 animals identically treated.

distributions following immunization and antigenic challenge were not significant for any one leukocyte type. Eosinophil, mast cell, and mononuclear leukocyte populations of peritoneal fluid and eosinophil and mast cell distribution in sections of lung and intestine did not differ significantly from those of unmanipulated frogs or those in control groups.

DISCUSSION

The ability of amphibian species to manifest an antibody response to the stimulus of particulate organisms has been established in studies involving toads²⁰ and frogs.^{26, 29} However, for soluble purified proteins an apparent requirement for antigenic enhancement has been demonstrated, e.g., the use of Freund's adjuvant in studies with the bullfrog, *Rana catesbeiana*,^{18, 21, 23} and the toad, *Xenopus laevis*,²⁴ or with bacterial cells in the marine toad, *Bufo marinus*.³⁶ Responses in amphibian models, however, may in turn depend upon physiologic differences and environmental factors related to native habitats of ectotherms. For in *Bufo marinus*, a tropical animal, antibody production followed a prolonged period of immunization with a protein antigen only at

relatively high temperatures.¹⁹ Apparently, injected foreign serum proteins in the toad^{36, 37} or frog³⁸ may remain in sustained blood concentrations for as long as 6 months. Thus, soluble antigen not readily taken up by their phagocytes may never attain sufficient contact with antibody-forming sites.³⁸ However, the natural antigenicity of microorganisms beyond their particulate character may serve as one critical factor in such less-advanced animals. Following injection of BGG adsorbed to the particle surfaces of 0.083 μ diameter monodisperse polystyrene latex (Dow), we were unable to detect corresponding serum antibody.

We have not yet been able to establish exact reasons for failure to raise agglutinating antibody to *S. typhosa* in some frogs, although there is suggestion of possible immune paralysis and/or tolerance. However, in this manifestation of immunoresponsiveness, as well as the hypersensitivity-type reactions reported here, factors of seasonally related and individual physiologic variability among frogs merit consideration. Cyclic changes of hibernation and summer physiologic functional states in *Rana pipiens* are not solely dependent upon the influences of induced external temperature variations. In addition, the nutritional states of gravid females and of males can differ under situations of stress. Thus it is admittedly difficult to be sure that all experiments begin with animals of uniform states of physiologic function and reactivity. All frogs utilized were therefore held for a minimum of four weeks under uniform conditions of housing and feeding to insure their states of health and nutrition and again after segregation under the particular environmental conditions required for subsequent experimental procedures. In addition, we were able to subsequently duplicate our experimental findings at random in both males and females received from the supplier at all seasons of the year.

That soluble cellular fractions, e.g., lipopolysaccharide, can react with serum antibody raised by injections of whole organisms was demonstrated in vitro. The challenge experiments in immunized frogs suggest that similar antigen-antibody complex formation occurs in vivo with ensuing anaphylactic manifestations as in mammals,³⁹ since these results could not be duplicated with whole microorganisms washed free of related soluble antigen. In those frogs that reacted to an initial injection of *S. typhosa*, we can only presume that prior natural exposure to an antigenically related microbial fraction may have been responsible for preformed antibody of sensitizing potential. In the case of BGG, despite the evidence for the presence of corresponding hemagglutinating antibody and precipitin formation, unequivocal shock reactions were demonstrated in only 2 out of 36 immunized frogs. Such limitations of antigenic efficacy may explain the previously reported failures to induce anaphylactic sensitization in frog experiments based solely upon trials of foreign sera.^{13, 14} Thus, anaphylactic sensitization in the frog may represent an early phylogenetic form since the character of antigen and serum antibody involved may differ from their counterparts responsible for hypersensitivity mechanisms in the more advanced homeothermic mammals.

The passive sensitization experiments are of interest and germane to this observation. Rabbit agglutinating antiserum to *S. typhosa* with hemagglutinat-

ing properties for the bacterial lipopolysaccharide and precipitin to an undetermined cellular fraction sensitized the frog to antigenic challenge. However, rabbit antiserum to BGG containing both precipitins and a hemagglutination titer equivalent to that demonstrated in actively immunized frogs was ineffective in transferring anaphylactic sensitizing properties. Whether this difference can be attributed to lack of appropriate receptors for binding the heterologous species antibody to specific tissue sites in the frog or to an inadequacy of foreign serum protein or resultant antigen-antibody complexes remains to be determined.

Anaphylactic response in an ectothermic animal provided an opportunity to observe some effects of evolutionary adaptability to environmental influences. Though reduced body temperature can favor a decrease in antibody production or release^{26, 30} in frogs, responses to antigenic challenge were not found to be correspondingly temperature dependent. Once immunization had been initiated, the subsequent development and course of sensitization were apparently not impeded by the altered respiratory-cardiovascular dynamics of hibernation at 4° C.

Total immersion of frogs required their dependence on skin function for respiration during anaphylactic responses. Symptoms in this group were uniformly marked and bypassing lung function did not affect pulmonary findings. We have not been able to identify a primary shock organ or affected tissue sites in either histamine shock¹³ or anaphylactic reactions in the frog. However, the nature of the manifestations in both these instances suggests the possibility of effects at some level of the nervous system.

While interrelationships between hypersensitivity manifestations, eosinophil leukocytes, and antibody classes are frequently noted, a common denominator of etiologic significance for all species studies has yet to be established. In frogs we have found eosinophilia a variable occurrence and not a related factor in the development of anaphylactic responsiveness.

In man, eosinophilia of affected tissue sites or related secretions is most commonly associated with reaginic antibody (IgE)-mediated atopic diseases (e.g., allergic rhinitis and asthma), specific drug allergy (e.g., serum sickness, penicillin reactions),⁴⁰ and parasitic infections characterized by immediate intracutaneous whealing responses to antigenic challenge (e.g., trichinosis, schistosomiasis, echinococcosis, filariasis). Lower in the phylogenetic scale eosinophilic activity of guinea pig antiserum has been identified in the 7S γ_1 fraction.⁴¹ However, in one model we have demonstrated that eosinophil cell responses could be effected by immune complexes with either 7S or 19S antibody moieties.⁴² In the case of anaphylaxis, 7S immunoglobulins have also been implicated: γ_1 for the guinea pig²⁵ and γ_2 in the rabbit.⁴³ It is therefore relevant to consider the evolutionary development of hypersensitivity and to note the associated findings of eosinophilia, anaphylactic manifestations, and a primary phase antibody response to bacterial antigen in an amphibian.

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