MFR PAPER 1287

Cellular Immunity in Fish as Measured by Lymphocyte Stimulation

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ABSTRACT—Fish are capable of responding to a variety of antigens. In many instances the primary response has attributes similar to those of mammals or birds, but in other ways the immunologic responses of fish stand apart, e.g., fish produce only one major class of immunoglobulin (IgM). Fish appear to handle all antigens as if they were thymus-independent. Some species have a faulty or nonexistent immunologic memory. Although fish lymphocytes perform certain functions characteristic of T or B cells of mammals, there is no clear-cut evidence that these are performed by specialized lymphocytes as opposed to lymphocytes with multiple functions. Several factors appear to be capable of regulating immune response. These include the IgM natural antibodies (some of which have nonspecific immunologic reactivity), immune complexes, and suppressor cells. All of these may combine to suppress certain responses. It therefore behooves the profession to undertake more extensive and intensive studies in fish immunology if the profession is to develop a better understanding of optimal modes and conditions for achieving protective immunity in fish.

Improvements and refinements in methodologies of tissue culture, immunology, virology, and bacteriology have made possible the attainment of new knowledge regarding the diseases of fish. A deeper appreciation of the factors contributing to the health of these poikilothermic animals has come from immunological studies. More recently, various parameters of cell-mediated immunity have come under scrutiny. It has been known for some time, and confirmed in our laboratory, that bony fishes are capable of rejecting allografts (Hildemann, 1957; Hildemann and Haas, 1960; Hildemann and Cooper, 1963). The rejection process in these animals is fairly acute. A more chronic process occurs in the shark (Hildemann, 1970). Allograft rejection in higher forms is mediated by lymphocytes activated by histocompatibility antigens which can be demonstrated in vitro by the blastogenic reaction. This is a complex reaction which is manifested in a variety of ways — increased permeability of lymphocyte membranes and elevation of the rate of synthesis of protein, RNA, and DNA. The biochemical changes are accompanied by an enlargement of cells whereby the lymphocytes revert to lymphoblasts, which can then reproduce by mitosis.

All these changes can be measured by biochemical or morphological methods, and the most commonly employed method measures DNA synthesis (incorporation of radioactive thymidine into DNA). Blastogenic transformation reactions occur as a result of activation of lymphocytes by histocompatibility antigens (antigens involved in graft rejection), as just mentioned, or as a result of lymphocyte stimulation by specific nontissue antigens, i.e., viral, bacterial, etc., or nonspecific mitogenic lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) or certain carbohydrates or lipopolysaccharides (LPS) in bacterial endotoxins.

Antigens can bind to specific receptors present on a few lymphocytes in the nonimmunized host and to the clones of lymphocytes following immunization with this antigen. This is believed to be the mode of expansion of a clone of lymphocytes specific for the antigen, assuming that the antigen is bound to the recognition receptor on the lymphocyte membrane, and this event generates a signal for a blastogenic transformation culminating in lymphocyte proliferation. The progeny lymphocytes recognize and respond (with blastogenic transformation) to the same antigen that launched the initial selective stimulation of the progenitor lymphocyte. This is a simplified version of an immunologic pyramidal reaction which does not take into account a variety of regulatory factors: proliferative asymmetry, suppressor cells, temporary anergy, etc. Moreover, knowl-

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edge about blastogenic transformation is derived mainly from in vitro studies, and relatively little is known about its occurrence and character in vivo. While blastogenic transformation induced by an antigen expresses a specific response, blastogenic transformation evoked by lectins or LPS is a more general response of large segments of lymphocytes regardless of specificity, commitment, or immune status. The only rule that seems to apply here is that T lymphocytes respond to PHA and Con A, and B lymphocytes react to LPS.

Following this introduction to blastogenic reactions let me return briefly to my initial comments about allograft rejection. As has been stated, fish reject allografts, and some fish reject them as rapidly as do mammals, but the analogy does not appear to extend to the blastogenic reaction. When the lymphocytes of two unidentical mice, A and B, are placed in culture they will react blastogenically — A VS B and B VS A. In contrast, when the lymphocytes of two snappers, A and B, are placed in culture they do not react in this manner. The reason for this discrepancy is not known. However, fish lymphocytes do respond with blastogenic reaction to certain antigens, and I will discuss this after a brief statement about the immunoglobulins and an overview of the immunologic response in fish.

IMMUNOGLOBULINS

We and others have shown that fishes, except for the lungfishes, possess only one kind of immunoglobulin, IgM (Clem and Sigel, 1963; Fish et al., 1966; Clem et al., 1967; Marchalonis and Edelman, 1968; Pollara et al., 1968). However, this single class of immunoglobulin exists in several physical states. It may exist as a 7S monomer, as a 14S tetramer, or as a 19S pentamer. To our knowledge, no one has conclusively shown the presence of other immunoglobulins, such as IgA, IgG, or IgD in fishes (except perhaps lungfishes). This was the main reason that William Clem and I were led to think that fishes were immunologically simple. But, as it developed in the course of our studies, even this single major immunoglobulin class has presented some rather challenging and interesting problems. I shall not go further into descriptions of immunoglobulins as this has been done in numerous reviews (Sigel et al., 1970; Clem, 1971; Hildemann and Clem, 1971; DuPasquier, 1973; Sigel, 1974). In this presentation I shall be more concerned with immunologic memory, regulation of the immune response, and cellular immunity.

IMMUNOLOGIC RESPONSE

By and large, the primary immune response is relatively efficient. One can evoke significant primary responses to many antigens provided the temperature is conducive or permissive to immunization. It has been recognized for a long time that the efficiency of immunization depends on the temperature of the water. Some very elegant studies on this problem have been conducted by Avtalion et al., (1973). Assuming that the temperature of the water is optimal or close to optimal, one can expect positive responses to primary immunization. As regards immunologic memory, the problem becomes more complicated. With some antigens, and in some species, there is a strong secondary response (Ridgeway et al., 1966). On the other hand, there are fishes which fail to respond to secondary stimulation. For example, in the sharks studied in our laboratory it was very difficult to elicit a secondary immune response at 1 month, 3 months, or 9 months after a successful primary immunization (Sigel and Clem, 1966). Only when the primary response was weak was it possible to elicit a heightened secondary response. If the primary response was strong, the secondary response would usually lack vigor, intensity, and amplitude.

Why this deficiency? Inability to make IgG has been suggested as an explanation, but teleosts are quite competent in mounting anamnestic responses even though they, too, fail to form IgG. The absence of a differentiated thymus gland is another possible explanation. Fishes do possess thymic glands, but these are rather primitive organs, resembling lymph nodes, and are virtually devoid of epithelial structures. In some ways the shark's immunologic response resembles the mammalian response to a thymus-independent antigen. One of modern immunology's dogmas holds that antibody production is a funtion of B cells — a class of lymphocytes characterized by their ability to synthesize immunoglobulins. Contained in this dogma is the precept that this B cell function requires the cooperation and help of thymus-derived lymphocytes, the T cells. But this is not an absolute requirement and there exist antigens which apparently succeed in stimulating B cells toward antibody production without the help of T cells. These are designated as thymus-independent antigens, in contrast to those which require helper T cells. One such antigen is pneumococcus polysaccharide. This thymus-independent antigen does not evoke a true secondary response in mice (Baker et al., 1970). It is tempting therefore to speculate that, in the absence of a mature (differentiated) thymus gland, any antigen can direct the immune mechanism in a manner analogous to the direction provided by thymus-independent antigen. What I am proposing is that, on the one hand the shark lacks helper T cells, and on the other that its B cells seem to respond to antigens which in higher animals require the helper function of T cells. This would imply that lymphocytes of fishes (at least some fishes) may not fit precisely into the categories created for mouse or human T and B cells. Moreover, it is possible that in phylogenetically lower classes some lymphocytes may perform both T and B functions or at least some of these functions.

REGULATION OF IMMUNE RESPONSE

One of the regulatory mechanisms in fish immunity probably resides in natural antibodies with reactivity directed to a variety of antigens. The shark is remarkable in this respect. The natural antibody, although an immunoglobulin and constructed like the typical 19S IgM antibody molecule, differs from the antibody raised by im-

munization in that it possesses an unusually broad specificity (Sigel et al., 1970). For example, antibodies prepared in rabbits by immunization against chicken red blood cells (CRBC) react with CRBC but not sheep RBC (SRBC) owing to narrow specificity and the lack of detectable cross-reactive antigens. The natural antibodies of sharks, on the other hand, react with a large array of RBC's — human, pigeon, chicken, rabbit, sheep, and even such exotic animals as the tapir. These antibodies also kill bacteria and neutralize viruses of humans (influenza) and chickens (Rous sarcoma). They also bind small haptens (Sigel et al., 1970). The origin of this polyreactivity is not known. What is even more remarkable is that the multiplicity of reactivity is resident in single antibody molecules. That is to say that individual molecules of 19S IgM react with multiple antigens. One way to show this is by mixed hemagglutination reaction as illustrated in Figure 1. Serum from an unimmunized shark is added to CRBC and the natural antibody is allowed to bind to the cells. After a short incubation, the cells are washed to remove free antibody and are mixed with SRBC which have not been exposed to antibody. The occurrence of agglutinated clumps or rosettes wherein CRBC attract on to themselves SRBC indicates that the antibody on the CRBC also forms a linkage with SRBC. This signifies that the shark natural antibody can bind to at least two distinctive antigens. Such dual specificity is usually not observed in antibodies raised by immunization. Other types of determinations based on antibody isolation by means of immunoadsorbents have led to similar conclusions: the natural antibody of the shark possesses polyspecificity. Such antibodies are likely to modify or regulate the immune response. They may deplete antigen to subimmunogenic levels. Alternately, natural antibody could conceivably change the physical state of the antigen, i.e., degree of dispersion or type of configuration rendering it more or less immunogenic. However, the most profound regulatory action is probably exerted by antibody-antigen complexes which will be discussed later.

EVIDENCE THAT ANTIBODIES CAN REGULATE LYMPHOCYTE RESPONSES

Studies aimed at elucidating the role of antibody in regulating lymphocyte functions were conducted in a large series of experiments based on blastogenic transformation reactions of lymphocytes of sharks, snappers, and rabbits. In these studies, we principally used specific antibodies raised by immunization. The response of lymphocytes from immune sharks to specific antigens is illustrated in Figure 2. Shark No. 5520 was immunized with bovine gamma globulin (BGG) and shark No. 7201 with poliovirus. Peripheral blood lymphocytes were cultured at 24°C in the presence of different concentrations of BGG or poliovirus. The lymphocytes of shark 5520 reacted specifically to BGG but not to poliovirus whereas the lymphocytes of shark 7201 reacted to poliovirus but not to BGG. A mixture of both antigens caused stimulation of lymphocytes in both sharks but the response was not increased above the level achieved by a single antigen.

Lymphocyte activation by specific antigen could be inhibited by antibody. This is illustrated in Figure 3. First, it should be noted that the lymphocytes of a shark immunized to BGG reacted to 100 μg and 10 μg of the antigen, but not to 1,000 μg. Thus, excess antigen was inhibitory. This may be viewed as a form of in vitro tolerance. Antibody to BGG prevented lymphocyte response to the stimulatory doses of 10 μg and 100 μg and did not reverse the "paralyzing" effect of 1,000 μg.

Cellular immunity to rubella virus antigens was studied extensively in the snapper. Several preparations of virus produced in different cell substrates were used for immunization and for stimulation of lymphocytes in vitro in order to obviate the problem of evoking blastogenic responses to cellular antigens. This would have occurred if the same viral antigen, e.g., virus produced in rabbit kidney cells were used for both immunization and in vitro testing. Lymphocytes from peripheral blood, thymus, anterior kidney, and spleen were cultured in the presence of different concentrations of rubella

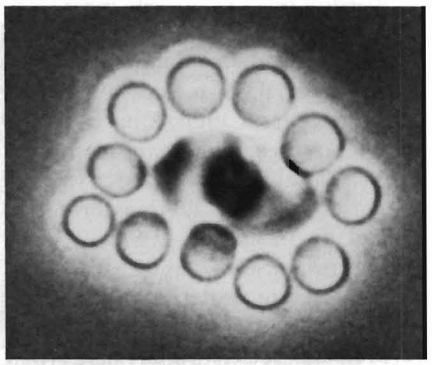

Figure 1.—Mixed hemagglutination reaction. In the center is a chicken red blood cell (RBC) and surrounding it are sheep RBC's. Shark natural antibody was mixed with the chicken RBC and subsequently washed to remove free antibody leaving only antibody bound to the chicken RBC. The attraction of sheep RBC is interpreted as indicating multispecificity of antibody.

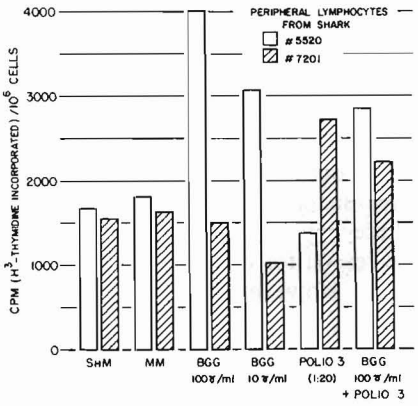

Figure 2.—Blastogenic response of immune shark lymphocytes to specific antigens. The test measures the incorporation of radioactive thymidine which denotes DNA synthesis evoked by the antigenic stimulation. ShM (shark growth medium) and MM (maintenance medium) are background controls indicating innate uptake of thymidine by unstimulated cells.

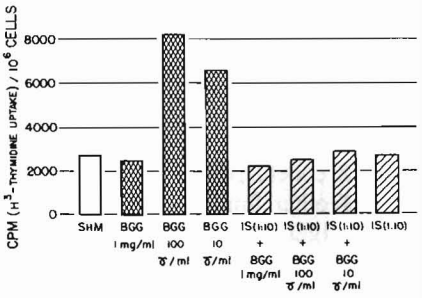

Figure 3.—Differences in response of shark lymphocytes to varying doses of BGG and the inhibition of the response by specific shark anti-BGG serum.

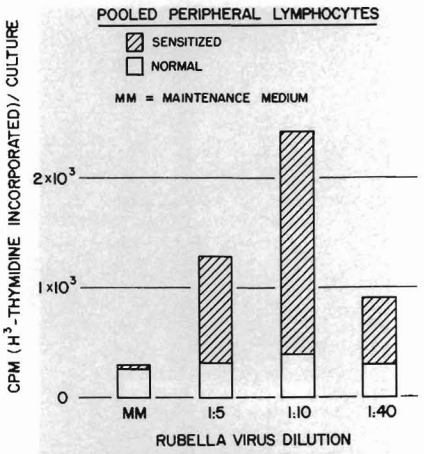

Figure 4.—Blastogenic response of snapper peripheral blood lymphocytes to rubella virus. The hatched columns represent results of lymphocytes from immunized snappers. The solid columns represent the results with lymphocytes from nonimmunized snappers.

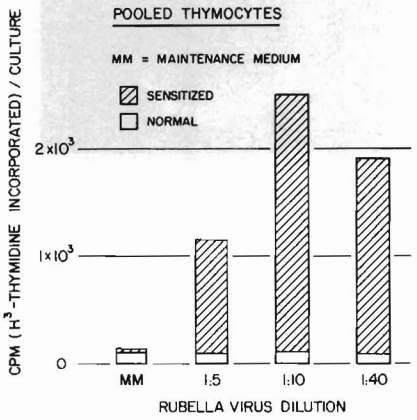

Figure 5.—Blastogenic response of snapper thymocytes to rubella virus.

virus. The results are presented in Figures 4, 5, and 6. In all instances there were significant responses of lymphocytes from immunized fish to the antigen as measured by thymidine incorporation. One can observe in the figures an optimal dose effect. Lymphocytes from nonimmunized snappers showed no response to any concentration of virus. These blastogenic transformation reactions could be abrogated by the addition of snapper antibody directed against rubella virus. This is shown in Table 1.

Thus, in the experiments with sharks and with snappers, antibody was inhibitory to the lymphocyte transformation response. These results were of

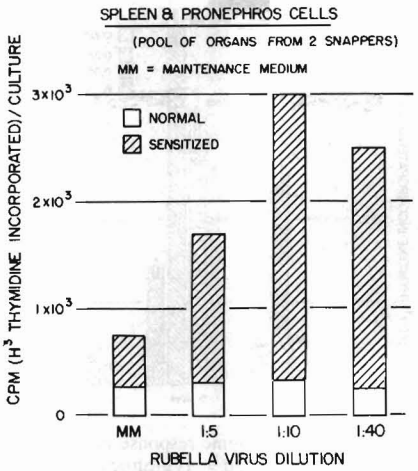

Figure 6.—Blastogenic response of snapper spleen and anterior kidney to rubella virus. Although in this experiment the spleens and anterior kidneys were pooled, in other experiments responses were observed with lymphocytes from separate organs. Furthermore, it has been possible to pool tissues from different snappers without causing allogenic responses.

special interest in view of the fact that studies in other systems have failed to demonstrate inhibition of antigen induced lymphocyte responses by antibody (Rosenberg et al., 1972). In other experiments, the dominant antibody appeared to be IgG whereas the fish antibody was IgM, and this fact may have accounted for the difference. These findings have led us to inaugurate a project on the effect of different classes of antibody on the lymphocyte response to rubella virus.

DIFFERENCES IN THE EFFECTS OF IgM AND IgG ANTIBODIES ON BLASTOGENIC TRANSFORMATION AND THE EFFECT OF IMMUNE COMPLEXES

In order to measure differential effects of different classes of immunoglobulin on the blastogenic response to a viral antigen, experiments were undertaken with lymphocytes and different classes of immunoglobulin from rabbits immunized with rubella virus. These data have been published (Lee and Sigel, 1974), but are being reviewed now for the sake of completeness. In Table 2, findings are presented which illustrate the ability of rabbit

Table 1.—Effect of antirubella serum on the blastogenic reaction of sensitized snapper lymphocytes to rubella.

	Fish #8807		Fish #8808	
Stimulant	cpm[1]	S.I.[2]	cpm	S.I.
None	236	—	309	—
Culture fluid control	309	1.31	371	1.20
Rubella virus (1:10)	1,527	6.47	1,554	5.03
Ab[3] (1:10)	309	1.31	411	1.33
RV[4] (1:10)+Ab (1:10)	302	1.28	535	1.73
RV (1:10)+Ab (1:40)	503	2.13	572	1.85
RV (1:10)+Ab (1:160)	717	3.04	510	1.65
RV (1:10)+Ab (1:640)	850	3.60	658	2.13

[1]Counts per minute per culture; mean of triplicate cultures.
[2]Stimulation index = cpm in stimulated cultures/cpm in unstimulated cultures.
[3]Snapper antirubella serum, minimum titer haemagglutination inhibition 1:320.
[4]Rubella virus.

Table 2.—Effect of rabbit antirubella serum on stimulation of immune rabbit peripheral blood lymphocytes by rubella virus.

Stimulant	cpm ± SD[1]	S.I.[2]
None	459± 17	—
Control[3]	839± 24	1.83
RV (1:10)[4]	4,415±212	9.62
RV+Ab[5] (1:10)	1,648± 96	3.59
RV+Ab (1:20)	2.213±121	4.82
RV+Ab (1:40)	2,474±115	5.39
RV+Ab (1:80)	3,226±202	7.03
RV+Ab (1:160)	4,410±245	9.61
RV+Ab (1:320)	4,392±213	9.57
Ab (1:10)	495± 12	1.80
PHA[6]	8,996±510	19.60

[1]Counts per minute per culture; mean of triplicate cultures ± standard deviation.
[2]Stimulation index.
[3]Extract of noninfected cell cultures.
[4]Rubella virus.
[5]Rabbit immune serum, heated at 56°C for 30 minutes before use.
[6]Phytohemagglutinin 0.01 ml (Lee and Sigel, 1974).

Table 3.—Differential effects of immune immunoglobulin-virus complexes on thymidine uptake by sensitized rabbit lymphocytes.

	Immune rabbit PBL		Normal rabbit PBL	
Stimulant	cpm±SD[1]	S.I.[2]	cpm±SD	S.I.
None	987± 28	—	1,056± 42	—
Control[3]	831± 49	0.84	1,014± 57	0.96
RV (1:40)[4]	13,626±952	13.81	1,122± 39	1.06
IgM[5]	999± 34	1.01	1,302± 62	1.23
IgG[6]	963± 24	0.97	1,350± 54	1.28
IgM+RV (1:40)	861± 62	0.87	1,188± 72	1.13
IgC+RV (1:40)	10,260±782	10.39	1,290± 45	1.22
PHA[7]	21,411±612	21.70	15,036±824	14.24

[1]Counts per minute per culture; mean of triplicate cultures ± standard deviation.
[2]Stimulation index.
[3]Extract of noninfected cell cultures.
[4]Rubella virus.
[5]Pooled and concentrated immune 19S immunoglobulin off Sephadex G-200, adjusted to contain 0.1 $O.D._{280}$ units/ml. This IgM preparation had a haemagglutination inhibition titer of 20 VS rubella virus.
[6]Pooled and concentrated immune 7S immunoglobulin off Sephadex G-200, adjusted to contain 0.1 $O.D._{280}$ units/ml. This IgG preparation had a haemagglutination inhibition titer of 40 VS rubella virus.
[7]Phytohemagglutinin 0.01 ml (Lee and Sigel, 1974).

immune serum to inhibit the response of rabbit immune lymphocytes to rubella virus. The differential effect of antirubella IgM and antirubella IgG is shown in Table 3. It is clear from this table that the IgM antibody suppressed

Table 4.—Effects of rubella virus-antibody complexes on the response of normal rabbit lymphocytes to PHA.

Stimulant	cpm ± SD[1]	S.I.[2]
None	721 ± 30	—
Control[3]	763 ± 35	1.05
PHA[4]	11,411 ± 590	15.82
RV (1:40)[5]	801 ± 8	1.11
RV + PHA	10,036 ± 113	13.91
IgM[6]	801 ± 18	1.11
IgM + RV	844 ± 42	1.17
[IgM + RV][7] + PHA	1,942 ± 150	2.69
IgG[8]	715 ± 20	0.99
IgG + RV	1,382 ± 38	1.92
[IgG + RV][7] + PHA	12,020 ± 342	16.67

[1]Counts per minute per culture; mean of triplicate cultures ± standard deviation.
[2]Stimulation index.
[3]Extract of noninfected cell cultures.
[4]Phytohemagglutinin 0.01 ml.
[5]Rubella virus.
[6]Pooled and concentrated immune 19S immunoglobulin off Sephadex G-200, adjusted to contain 0.1 $O.D._{280}$ units/ml. This IgM preparation had a haemagglutination inhibition titer of 20 VS rubella virus.
[7]Mixtures of fractionated antibodies with virus were incubated at 37°C for 45 minutes after which they were added to lymphocyte cell cultures. The cultures were then put in a CO_2-enriched atmosphere at 37°C for 15 minutes before PHA was added.
[8]Pooled and concentrated immune 7S immunoglobulin off Sephadex G-200, adjusted to contain 0.1 $O.D._{280}$ units/ml. This IgG preparation had a haemagglutination inhibition titer of 40 VS rubella virus (Lee and Sigel, 1974).

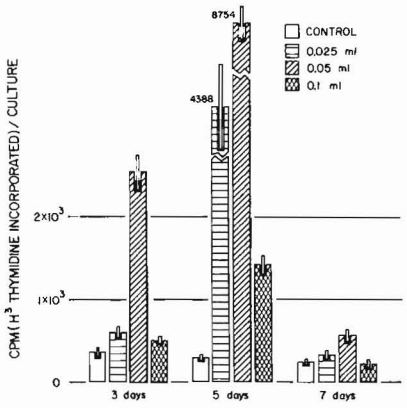

Figure 7.—Blastogenic response of snapper peripheral blood lymphocytes to phytohemagglutinin (PHA). Control refers to lymphocyte cultures to which PHA was not added. The other columns show responses to different amounts of PHA as shown in the legend in the upper right.

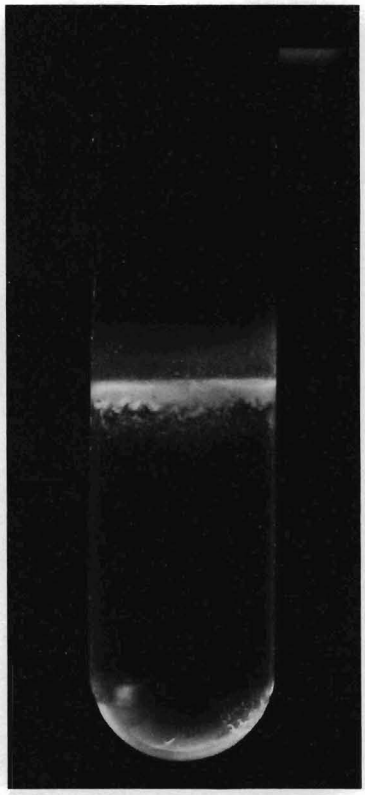

Figure 8.—Separation of shark lymphocytes on Ficoll-Isopaque. Note the three layers of cells in the middle of the tube. An additional cell population of lymphocytes is located at the bottom of the tube.

the response whereas the IgG did not. These findings are in accord with the inhibitory effect of fish antibodies which belong to the IgM class.

In an attempt to gain insight into the mechanism of inhibition of blastogenic transformation, we have performed experiments in which the virus was allowed to complex with specific antibody, IgG or IgM, the immune complex was added to lymphocytes, and their response to mitogen PHA was determined. The results are given in Table 4. It can be seen that the IgM-antigen complex blocked the response of lymphocytes to PHA; the IgG-antigen complexes exerted no inhibitory action. This suggests that IgM antibody may play a more dominant role in regulation of the immune response. This finding assumes special significance in view of the presence of natural IgM antibodies in fishes, notably in the sharks, and it may explain, at least in part, the failure of sharks to mount a secondary immune response.

SUPPRESSOR CELLS

Still another mechanism of regulation is mediated by suppressor cells. These cells exert inhibitory effects against other lymphocytes upon activation by antigens, mitogens, or other factors. In higher vertebrates both T and B cells and probably monocytes are apparently capable of becoming suppressors (Gershon, 1974; Kirchner et al., 1974; Singhal et al., 1972). They dampen or stop immune responses in vivo and in vitro. It now appears from provisional findings that such cells also exist in fishes as evidenced by suppression of blastogenesis.

Snapper lymphocytes were shown to possess a relatively well-developed capability to react to plant mitogens. An experiment measuring the response of snapper peripheral blood lymphocytes to PHA is given in Figure 7. It can be seen that a fair response to PHA was evident in cultures at 3 days and a maximum response at 5 days. In contrast were the findings with lymphocytes from nurse sharks: here it was difficult to elicit a response to PHA, and the responses to Con A required high doses of mitogen. Although these responses are nonspecific as they do not reflect sensitivity to a specific antigen, they nevertheless are considered indicative of the overall immunologic status (or development) of the host. The findings would therefore imply some kind of deficit in the shark, presumably attributable to T cells. Further investigations were therefore conducted to elucidate this problem. Shark lymphocytes were separated on Ficoll-Isopaque[1]. This procedure has permitted the separation and recovery of at least three subpopulations of lymphocytes as illustrated in Figure 8. The individual bands or pellet of cells (bottom) were subjected to blastogenic transformation reactions with Con A and PHA. Results in Figure 9 show that all three subpopulations respond to Con A. In Figure 10 the results with PHA are shown. It should be noted that most of the subpopulations of shark lymphocytes did not react to PHA, but a reaction was obtained with the bottom cells. In one experiment (not shown) it was possible to inhibit the response of the bottom cells to PHA by the addition of interphase or top cells.

We conclude provisionally that sharks possess PHA responsive cells, and also suppressor cells, which are

[1]Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

capable of inhibiting a function(s) of the responsive cells.

SUMMARY AND COMMENTS

1. The blastogenic transformation reaction is a useful indicator of immunity in fish. The ability of lymphocytes of sharks and bony fishes to react to specific antigens attests to a form differentiation observed in more advanced species, i.e., mammals. While specialized classes and subclasses of lymphocytes have been recognized in mammals, our present state of knowledge does not permit distinction in fish of true T or B cells and certainly not the subsets of T and B which perform different functions in mammals.

2. The blastogenic transformation reaction has demonstrated three modes of immunologic regulation: lymphocyte responses can be inhibited by IgM antibody, by IgM antibody-antigen complexes, and by suppressor cells.

3. Certain fishes possess natural antibodies with broad polyspecificity. These IgM immunoglobulins may have a tremendous survival value for the sharks. Yet, the same antibodies may be responsible for immunologic amnesia and perhaps for diminution in cell-mediated immunity in the shark.

More information is urgently needed on these and other matters for the sake of knowledge about the intelligent approaches to immunization of fish (and avoidance of disastrous effects) and for the sake of furthering our understanding of immunologic developments and functions as they relate to other animals, including man.

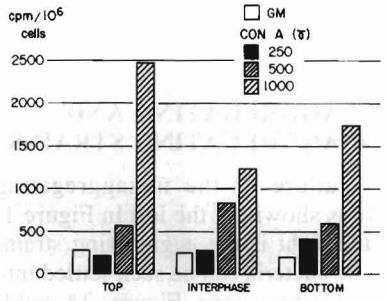

Figure 9.—Blastogenic response of shark lymphocytes to Concanavalin A (Con A) after separation on Ficoll-Isopaque.

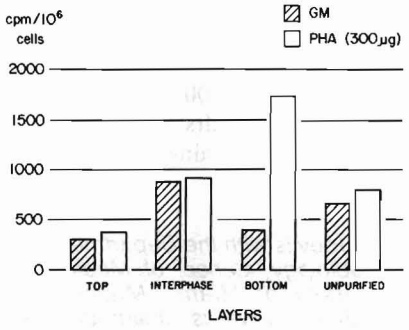

Figure 10.—Response of shark lymphocytes from the pellet (after separation on Ficoll-Isopaque) to Phytohemagglutinin.

ACKNOWLEDGMENT

Early work on these studies was supported by the U.S. Public Health Service Research Grant No. A105758 from the National Institute of Allergy and Infectious Diseases. Current work is being supported by the Sea Grant awarded to the University of Miami.

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