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REVIEW

Self or Non-self Discrimination in Ascidians

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Among the vertebrates, even the most primitive forms, the Agnathans possess a specific immune system with an antibody response and rejection of foreign grafts. Invertebrates, on the other hand, have long been considered to be lack of this system. However, recent studies concerning the phylogenetic development of immunity suggest that the origins of immunological reactivity lie within the invertebrates [1-4].

Ascidians, a group of protochordates, are situated in a point of contact between invertebrates and vertebrates. The vertebrates are genetically considered to be evolved directly from these animals [5]. In many groups of compound ascidians, a phenomenon exists which is analogous to transplantation specificity, known as colony specificity. All ascidians possess lymphocyte-like cells [6-10] and these cells exhibit some properties like vertebrate lymphocytes, such as X-irradiation sensitivity [11, 12], ability of forming rosettes with sheep erythrocytes [3] and lectin receptors on their surfaces [13]. Therefore, it is in ascidians that many immunologists expect to elucidate the origins of the specific immune system of the vertebrates.

ASCIDIAN IMMUNE-LIKE RESPONSES

Diverse ascidians respond to foreign materials by a number of cellular and humoral defense mechanisms. Humoral responses show the presence of natural agglutinins and anti-microbial factors. Cellular responses include phagocytosis, encapsulation, and contact reaction.

Humoral substances

One of the earliest work concerning ascidian humoral substances which exhibit immune-like response was carried out by Cantacuzene [14]. According to him, *Phallusia mamillata* can produce a nonspecific precipitin to rabbit serum after multiple injections into the endostyle sinus. Then, Tyler [15] reported that agglutinins to spermatozoa from some invertebrates existed in the sera of *Ciona intestinalis* and *Styela barnharti*. In recent years, agglutinins to mammalian erythrocytes have been found in some ascidians and some of these hemagglutinins have been partially characterized. These hemagglutinins are summarized in Table 1.

Cellular responses

Although Metchinikoff's idea on the importance of phagocytic cells in the defense mechanisms against pathogens has long been recognized as true for vertebrates, it has been ignored that such cells may also play a major role in the defense mechanisms of invertebrates. In recent years, however, the role of phagocytic cells in the defense mechanisms of invertebrates has been reexamined [cf. 24]. In the solitary ascidian, *Molgula manhattensis*, injected dyes were taken up by several types of phagocytic cells [25]. Blood cells of another solitary ascidian have been observed to phagocytize and eliminate sea urchin spermatozoa and fixed

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TABLE 1. Summary of the occurrence of agglutinins in some ascidians

Species	Response to	Reference
Ciona intestinalis	vertebrate erythrocytes	Wright and Cooper [16], Wright [17]
Ascidia ceratodes	invertebrate spermatozoa	Tyler [15]
A. malaca	rabbit & human erythrocytes	Parinello and Canicattì [18]
Botrylloides leachii	sheep erythrocytes	Coombe *et al.*, [19]
Styela plicata	mammalian erythrocytes	Fuke and Sugai [20]
Halocynthia hilgendorfi	mammalian erythrocytes	Fuke and Sugai [20]
H. pyriformis	mammalian & avian erythrocytes	Anderson and Good [21]
H. papillosa	human erythrocytes	Bretting and Renwrantz [22]
Microcosmos sulcatus	human erythrocytes	Bretting and Renwrantz [22]
Phallusia mamillata	human erythrocytes	Parinello and Canicattì [23]

mammalian erythrocytes [26].

When introduced foreign materials are relatively large in size, these are not phagocytized, but usually encapsulated. In ascidians, encapsulation occurs to isolate natural invaders [27–29] or experimentally inserted objects [25, 30–32]. Vacuolated blood cells, such as morula cells or vanadocytes, are responsible for these reactions [10, 25].

Recently, nonphagocytic cellular reaction called contact reaction has been reported in the solitary ascidians [33, 34]. According to Fuke [33], when isolated blood cells from two species were brought into contact *in vitro*, both cells reacted against each other, resulting in reciprocal lysis of both cells. Moreover, blood cells from two individuals of *Halocynthia roretzi* were found to exhibit the same type of cellular reaction depending upon the combinations.

COLONY SPECIFICITY IN COMPOUND ASCIDIANS

In 1903, Bancroft [35] reported an important and informative work on the compound ascidian *Botryllus schlosseri*. He described that colonies of different origin did not fuse together after grafting, regardless of the conditions. In fact, they rejected each other. However, fragments from any one single colony easily fused together. Some of the F_1 colonies developing from larvae released by one parental colony fused, but others did not. He attempted to explain these phenomena on the basis of a mechanism of fusion or non-fusion determined by inherent differences among different colonies of the species.

Following this interesting account, no similar or systematic research had been undertaken until we in Japan began experimental studies on fusion and non-fusion in the Japanese ascidian *Botryllus primigenus*.

1. General description of colony specificity

Occurrence of colony specificity

In *Botryllus primigenus*, when two pieces from the same colony were placed in juxtaposition at their growing edges, they fused and formed a common vascular system. If the faced edges were cut artificially, the fusion of two pieces occurred in the same way. This phenomenon was designated as "fusion". On the contrary, when the pieces were obtained from different colonies within the natural population, the contact of the pieces usually resulted in necrosis at the contact area of naturally growing edges or artificially cut surfaces. This phenomenon was designated as "non-fusion reaction" (NFR), in other words "rejection". These phenomena in which two colonies exhibit fusion or rejection are called colony specificity. Most species of the compound ascidians usually exhibit fusion or rejection when two colonies of the same species are made contact. In a certain species, e.g., *Polycitor mutabilis* [36] colony specificity seems to be absent. Neither the complete fusion of the test matrix nor the particular rejection between two colonies was observed at the

TABLE 2. Summary of the occurrence of colony specificity in some compound ascidians

Species	Contact with		Reference
	Growing edges	Cut surfaces	
Aplydium yamazii	F or R	F or R	Watanabe and Taneda [37]
Amaroucium constellatum	F or R	—	Freeman [38]
Polycitor mutabilis	I	F	Oka and Usui [36]
Didemnum moseleyi	F or R	F or R	Mukai and Watanabe [39]
Perophora viridis	F or R	—	Freeman [38]
P. japonica	F or R	—	Koyama and Watanabe [40]
P. orientalis	I	F	Mukai and Watanabe [39]
P. sagamiensis	F or R	—	Koyama and Watanabe [41]
P. bermudensis	F or R	—	Freeman [38]
Ecteinascidia tortugensis	F or R	—	Freeman [38]
Botryllus schlosseri	F or R	—	Bancroft [35]
B. primigenus	F or R	F or R	Mukai and Watanabe [39]
B. scalaris	F or R	F or R	Saito and Watanabe [42]
Botrylloides simodensis	I	F or R	Saito and Watanabe [43]

F, "Fusion"; R, "Rejection"; I, "Indifference"; —, No experiment was performed.

contact area. This phenomenon was designated as "indifference". On the contrary, when cut surfaces of two pieces were made contact, they always fused together regardless of their origin. Results of fusibility experiments obtained from some compound ascidians are summarized in Table 2.

Features of fusion and non-fusion reaction (NFR)

When two compatible colonies are brought into contact, fusion of colonies always occurs. From the electron microscopical studies concerning the fusion reaction between colonies in *B. primigenus*, it has been revealed that the ampullae from two colonies fused in the tip-to-side manner following the constitution of new intercellular junctions and the elimination of degenerated or healthy epithelial cells at the contact area [44]. Recently, fusion of tunic between two individuals has been reported in the solitary ascidian, *Molgula complanata* [45]. This finding suggests the presence of the ability to recognize the allogeneic differences between individuals in a solitary ascidian as well as in compound ascidians.

When two incompatible colonies of *B. primigenus* came into contact with the growing edges, rejection called non-fusion reaction (NFR), as stated before, took place at the contact area. The process of NFR is schematically illustrated in Figure 1. During the process of NFR, six stages were recognized [46]. These are as follows: Stage 1 (0 hr after the contact): The margins of test matrices of two colonies contacted with each other following the growth of the colonies. Stage 2 (1–2 hr after the contact): The tips of the ampullae pushed against each other and penetrated into the test matrix of opposite colony. No change was recognized in the contact area at this stage. Stage 3(4–5 hr after the contact): The penetration of ampullae into test matrix was further advanced and the first sign of NFR, or the increase in opacity occurred at the tips of the penetrated ampullae. In this stage, we have observed recently the infiltration of morula cells and possible increase in permeability of ampullar epidermal cells [47]. Stage 4 (7–8 hr after the contact): The ampullae of both colonies stopped further penetration into the test matrix of the opposite colony. Stage 5 (9–10 hr after the contact): The ampullae began to contract and to become thinner at their proximal parts, where blood flow decreased. Stage 6 (12 hr after the contact): The contraction of the ampullae further proceeded, and the blood stream in the ampullae stopped completely. The distal part of the ampullae were finally constricted off from the healthy parts of the proximal vascular system, and

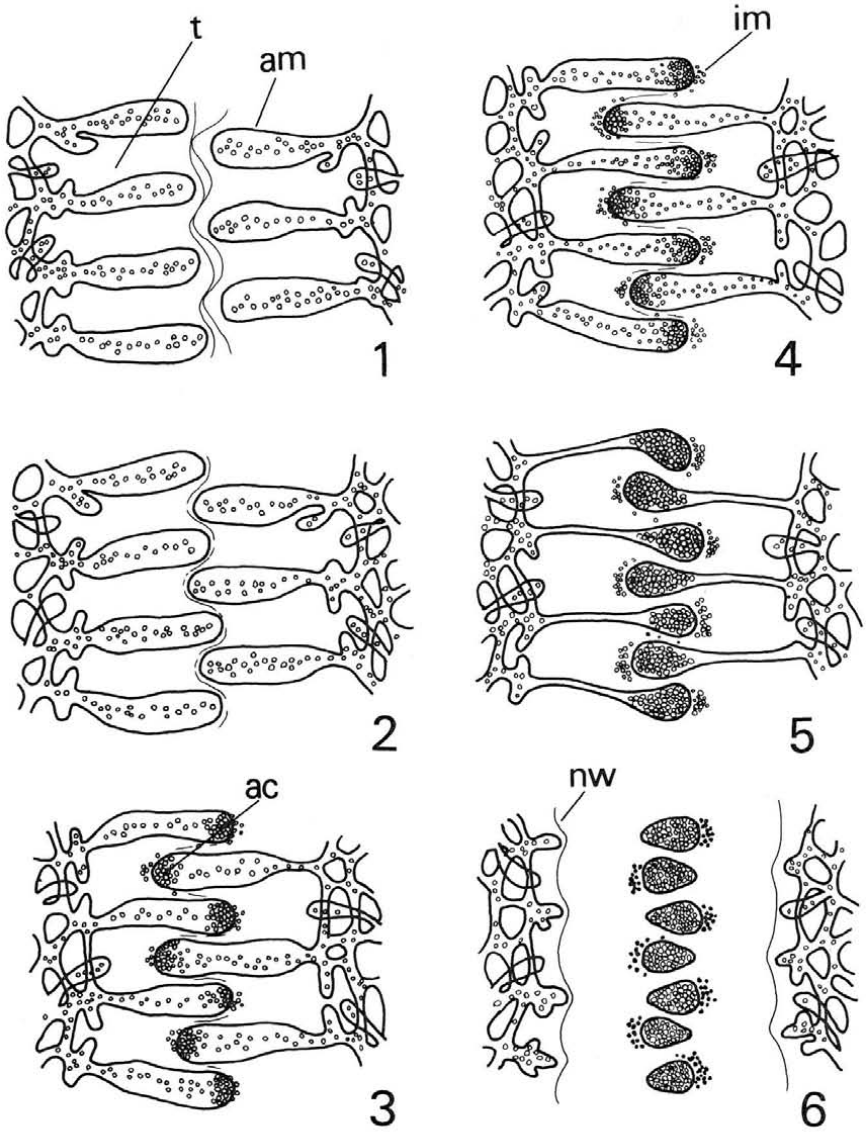

FIG. 1. Schematic illustration of the process of non-fusion reaction (NFR). Numbers indicate stages of NFR. ac, aggregate of blood cells; am, ampulla; im, infiltrated morula cells; nw, new wall; t, test matrix. (Permission by Am. Zool.)

new walls were formed to separate the necrotic zone from the healthy parts of both colonies. Electron microscopical studies revealed the destruction of test cells and the formation of filamentous structures in the test matrix in NFR, although no change was observed in the ampullar epidermal cells and blood cells retained in the vascular system [46].

In the case of xenogeneic contact with their growing edges, it has long been considere that no rejection like NFR took place [39]. Recently, however, we have demonstrated the presence of the xenogeneic rejection like NFR among the colonies of three Japanese botryllids, *Botryllus scalaris*, *Botryllus primigenus* and *Botrylloides simodensis* [48]. These three species show the colony specificity, though in different manners, respectively.

When a colony of *B. primigenus* or *B. simodensis* came into contact with a colony of *B. scalaris*, xenogeneic rejection like NFR always took place in the colony of *B. primigenus* or *B. simodensis*, while no rejection took place in *B. scalaris*. In the case of contact between cut surfaces, the xenogeneic rejection appeared within 2 hr after the contact, whereas in the case of that with growing edges, such rejection did not appear until 1 to 3 days after the contact.

When the colonies of *B. primigenus* and *B. simodensis* were made contact with their cut surfaces, each colony also showed the rejection like NFR about 6 hr after the contact. While, in the case of the contact between growing edges, no rejection appeared in both colonies.

Xenogeneic rejections in all combinations showed the same features, though different periods of time were needed for initiation of the rejection. These features are almost the same as those shown in allogeneic NFR.

With regard to the allogeneic recognition in three Japanese botryllids, *B. scalaris*, *B. primigenus*, and *B. simodensis*, the following matters have been confirmed. A colony of *B. simodensis* could distinguish a compatible colony from an incompatible one at its surface of tunic, when it contacted with another colony. Such a type of recognition can never be seen in other species. In *B. scalaris*, NFR did not occur until the fusion of vessels was established between two incompatible colonies.

From these findings, we have supposed that there are at least three steps of the allogeneic recognition in botryllids (Fig. 2).

The first step is the recognition at the test surface. This type of recognition is known only in *B. simodensis* at present. Electron microscopical studies on the fusion of tunic revealed that the tunic of botryllids consisted of two layers, highly electron-dense cuticle and translucent test matrix, and that the fusion of confronting cuticles was necessary to complete the fusion of tunic between two colonies [44, 49]. In *B. simodensis*, therefore, there seems to be a slight difference in cuticle structures to prevent fusion between compatible and incompatible colonies, while not in *B. primigenus* and *B. scalaris*.

The second step is the recognition of the allogeneic humoral factor(s) diffusing through the test matrix. This type of recognition is shown typically in *B. primigenus* and also shown experimentally in *B. simodensis* [39]. This is always accompanied with NFR.

The third step of the recognition takes place in vascular vessels. This was shown in *B. scalaris* [42], and may be fundamental ability in botryllids. They must have acquired higher level of recognition ability according to evolution.

2. Experimental analysis on the mechanism of colony specificity

Genetic control of fusibility in Botryllus

Oka and Watanabe [50–52] studied the fusibility between the colonies of *B. primigenus* taken from nature and among F_1 and F_2 progenies derived by the crossing of two incompatible colonies (in *B. primigenus* self-fertilization does not occur). The results obtained can be summarized as follows:

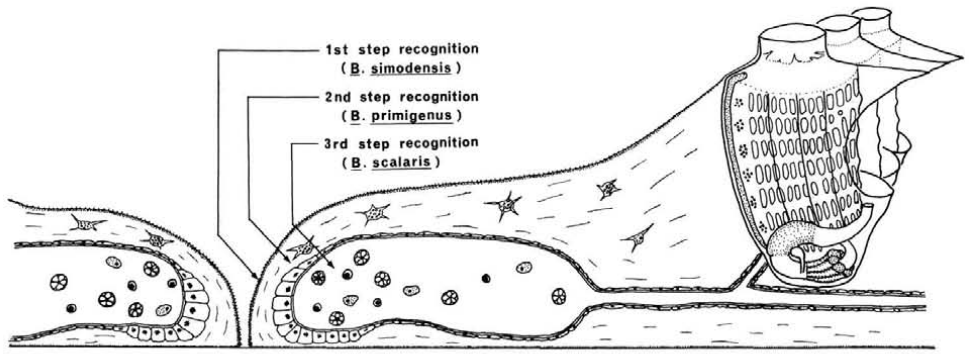

FIG. 2. Three steps of the allogeneic recognition in botryllids.

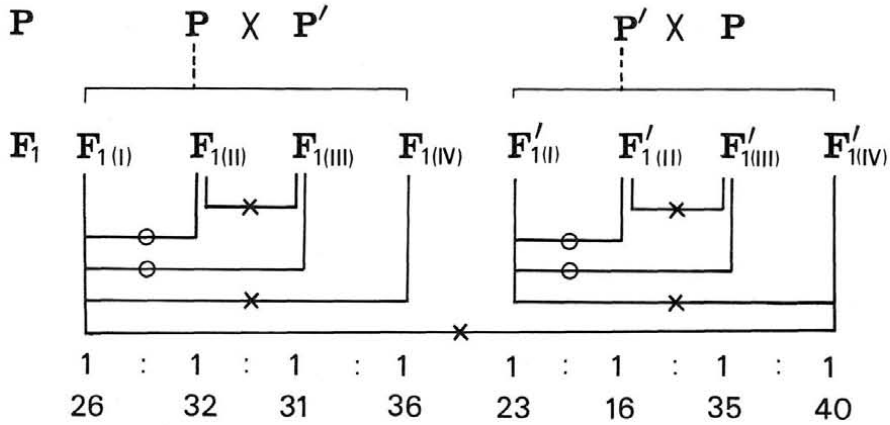

FIG. 3. Fusibility among F_1 colonies derived by the crossing of two incompatible colonies (P and P′). —○—, fusion; —×—, non-fusion.

(a) Almost all colonies taken at random from nature do not fuse with each other. (b) F_1 colonies are sorted into four groups, which appear in proportions of approximately 1: 1: 1: 1. Members of each group fuse with one another and with those of two other groups (Fig. 3). (c) Fusion is always possible between daughter colonies and the mother colony. (d) F_2 colonies obtaind by crossing of two incompatible F_1 colonies are also sorted into four groups in the same manner as in F_1.

In order to explain the apparently complicated fusion and non-fusion phenomena just outlined, it is necessary to introduce some hypotheses to bring all details into unified and simple relationship as described below. (1) The genes controlling fusibility among colonies are under natural conditions heterozygous. (2) They are similar to a series of multiple alleles of the type (S genes) which controls self-incompatibility in flowering plants. (3) Colonies sharing at least one common allele are mutually fusible with one another, while those containing no common allele are non-fusible. A schematic illustration of this hypothesis appears in Figure 4. On the basis of these postulates, it is possible to explain in reasonable terms the experimental results obtained.

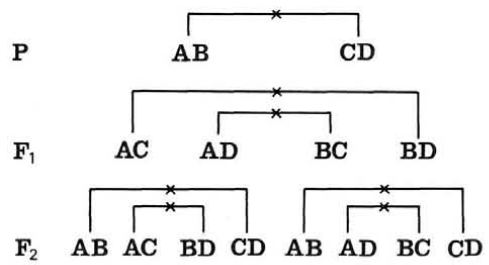

FIG. 4. Schematic diagram of the hypothesis on fusibility of *Botryllus primigenus*. ———, non-fusible; otherwise fusible. (Permission by Am. Zool.)

Let us consider first the case where four classes of colonies are produced from two incompatible colonies between which fertilization has been accomplished. The majority of colonies formed in nature can be represented according to a series of letters of the kind AB, CD, EF, ..., since they carry no common gene and are incompatible with one another. The crossing of AB with CD produces four classes of F_1 colonies which can be designated AC, AD, BC, and BD. All such individuals of course share common alleles with the parental colonies AB and CD, and are therefore fusible with them. Members of each of the four classes share common alleles with two of the remaining three classes, and are therefore fusible with them.

Any crossing among different, incompatible classes of the F_1 generation, such as AC and BD, produce four classes in the F_2 generation with fusibility of the same kind as that just described. However, on account of self-incompatibility the crossing of fusible colonies of different class, such as AC and AD, produces only two classes in each case (AD and CD from AC, and AC and CD from AD).

All pairs of different colonies selected from the three classes AC, AD, and CD have a single common allele, and so are fusible with one another. However, the lack of a common allele between AC and BD or AD and BC prevents their fusion. CD shares a common allele with both BC and BD and is thus fusible with them. To distinguish between the two classes of the F_2 generation produced by fusible F_1 colonies it is thus necessary to attempt fusion with members of other generations.

In order to examine both the validity of these ideas and the actual distribution pattern of alleles controlling fusibility in *B. primigenus*, fusion experiments were carried out on a large number of colonies collected from three areas of the Izu peninsula [53, 54]. No exception to the rules described above was found, thus implying very strongly that the fusibility among colonies of this species is in fact controlled by a series of multiple alleles at a single locus.

The results from recent studies with another *Botryllus* species were consitent with this hypothesis and suggested the idea that recognition of the product of a "self" allele is necessary for colony fusion [55, 56]. Scofield *et al.* [55, 56] claimed that *Botryllus* fusibility might be controlled by genes of an ancestral MHC gene complex.

Experimental alteration of fusibility

As mentioned above, the fusibility of *Botryllus* colonies is genetically controlled. Two pieces taken from the same colony and kept apart over a period of one year did not alter their original fusibility. Interestingly, however, the fusibility of the colonies can be altered by the techniques of fusion and re-separation of the colony containing only one allele in common [57]. For example, a colony BC was fused with a sister colony AC. Four days after fusion, the two colonies were separated again. Then, BC colony became non-fusible with BD colony which should be originally fusible. In the same manner, AC colony became fusible with BD colony by fusion and re-separation of BC colony. In the case of the alteration from fusible to non-fusible, when AC colony was larger than BC colony, acquired fusibility of BC (to become non-fusible with BD) remained unaltered. On the other hand, when BC colony was larger than AC colony, acquired fusibility of BC returned to original fusibility after certain periods of time. In Mukai's series I (non-fusible alteration) and series II (fusible alteration) experiments, relative sizes of the two (BC and AC) were varied. The smaller the relative size of AC colony to BC colony, the shorter the time required for returning to original fusiblity of BC (series I). In the same manner, the larger the relative sizes of BC colony to AC colony, the shorter was the mean time required for fusion to take place (series II). Therefore, it is concluded that the fusibility of *Botryllus* colonies depends upon blood in the colonies.

Mukai [57] recognized two steps in the alteration of fusibility, primary and secondary alterations. Primary alteration may be responsible for some substances in blood. The fusibility of a separated colony is determined by the relative concentration of substances originated from two colonies, fusibility altered and removed. The relative concentration results from merely the mixture of blood of two colonies. Secondary alteration is brought about by changes in the relative concentration of substances. Such an alteration may be responsible for the blood cells originating the substances.

Mechanism of NFR

On the basis of light and electron microscopical observations, Tanaka and Watanabe [46] tentatively interpreted that the first step of NFR took place in the test cells in the test matrix. Recently, we experimentally examined the validity of this hypothesis [37, 47]. All results obtained from these experiments suggested that the first step visible for NFR took place in the ampullar lumen. No evidence suggesting the participation of test cells was obtained.

As mentioned above, fusibility of colonies depends upon blood in the colonies [57]. This means that the agent(s) concerning fusibility is involved in blood. From the result of fusion among three F_1 strains (AC–BC–BD) performed by Tanaka [58], it can be easily interpreted that NFR takes place by the mixture of blood from two incompatible colonies. In other words, blood itself contains both an "effective agent" and a "recognition agent" concerning fusibility.

Recent work based on *in vivo* bioassay revealed

that the injection of allogeneic whole blood was sufficient to induce NFR [59]. The injection of syngeneic blood had no effect. Three possible combinations can be considered in the interaction between the blood from two incompatible colonies; (1) the interaction between blood cells; (2) that between blood cells and blood plasma; (3) that between blood plasma. Further investigations with blood fractions strongly suggested the participation of blood cells in NFR [59]. Therefore, the possibility of the interaction between blood plasma is negligible. Injection of allogeneic blood plasma induced weak positive responses. Studies on tunic interaction in *B. schlosseri* with the dye neutral red showed no cellular movement between two colonies in contact with each other [38]. These results led us to hypothesize that NFR was initiated by an interaction between blood cells and a humoral factor(s) in the plasma [59].

More recently, the specimen in which fusion and NFR simultaneously appeared was discovered [60]. This finding reinforces our hypothesis on the mechanism of NFR.

Electron microscopical studies in *B. primigenus* revealed that degenerated "self" cells which might be eliminated were always coated with microfibrous materials on their surfaces, while other healthy blood cells were not [61]. Katow and Watanabe [61] pointed out the possible role of these fibrous materials in discrimination of degenerated (=not self) cells. Such fibrous materials, though their features were not identical, were also found in intracellular spaces of a blood cell cluster formed in vessels of the central colony in the fusion with three F_1 colonies [58].

From these results we consider the mechanism of NFR in *B. primigenus* to be as follows (Fig. 5): A humoral factor(s) concerning NFR, or recognition molecule, exists in the test matrix as well as in the blood plasma. This factor may be originated from blood cells (=lymphocytes). When two incompatible colonies come into contact and the ampullae penetrate into the test matrix of opposite colony, uptake of this factor into the lumen of the penetrating ampullae occurs. This factor reacts only with non-self blood cells and the formation of a cluster of blood cells takes place in connection with an increase in permeability of the vessels and the infiltration of morula cells. The formation of the cluster results in the stoppage

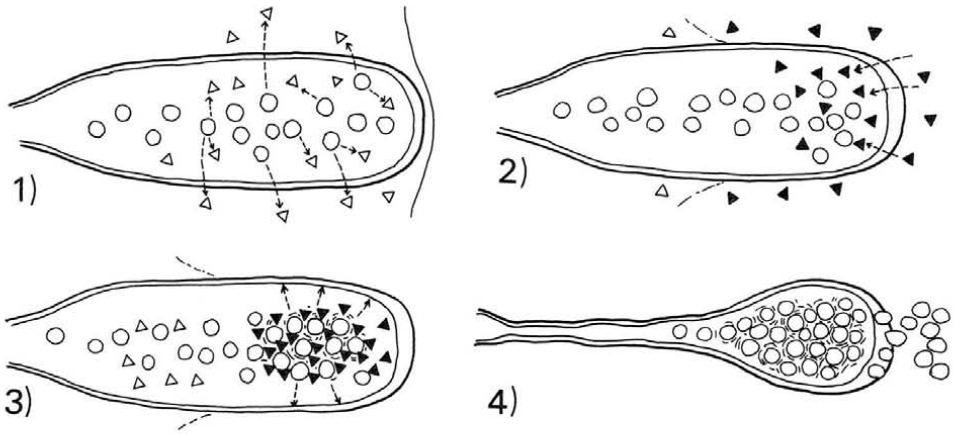

FIG. 5. Schematic presentation of a possible mechanism of NFR in *Botryllus primigenus*.

1) Lymphocytes excrete a humoral factor, or recognition molecule, into blood plasma and test matrix.

2) When the colony grows and an ampulla penetrates into the test matrix of opposite colony, the humoral factor in the test matrix of opposite colony invades into the ampullar lumen.

3) This factor reacts with non-self blood cells and the formation of a cluster of blood cells occurs. Then, reacting blood cells release a factor which exerts a contraction effect on the ampullar epidermal cells.

4) Then, the stoppage of blood flow takes place accompanying the infiltration of morula cells.

of blood flow and the constriction of the vessels. The contraction and the increase in permeability of the vessels may be responsible for some factors released from the reacting blood cells. The destruction of test cells and the formation of new wall occurs subsequently.

In *B. scalaris*, on the other hand, a colony showed no rejection against the blood plasma derived from an incompatible colony [62]. Therefore, in this species, the allogeneic rejection may responsible for the direct contact between incompatible blood cells just like the contact reaction of blood cells in some solitary ascidians [33, 34].

Nature of the agents participating in NFR

By the use of Mukai's fusibility alteration technique [57], we showed that a factor(s) concerning fusibility was sensitive to X-irradiation [12]. This result suggested that at least one factor concerning fusibility might be a cellular component in blood, supporting the conclusion obtained from the injection of blood fractions described before. Since lymphocyte population decreased in the irradiated preparations in which a factor concerning fusibility also reduced, these cells may play a role in colony specificity.

Recently, the first approach to the biochemical characterization of humoral factors responsible for the colony specificity in ascidians was tried by using blood plasma from colonies of *B. simodensis* [63]. As stated before, this species also shows colony specificity, and a remarkable NFR appears when two incompatible colonies are brought into contact with their cut surfaces.

The blood plasma of *B. simodensis* induced NFR-like responses in allogeneic challenges, although no response induced in syngeneic challenges, as well as in *B. primigenus*. This NFR-inducing activity in the plasma was retained after dialysis against filtered seawater, while almost activity was lost by heat higher than 55°C. The activity was dependent on the presence or absence of divalent cations, such as Ca^{2+} and Mg^{2+}. The specificity of the activity was easily lost even by the mild treatments, such as long-term storage at 4°C, freezing and thawing, incubation at a moderate temperature, and other physico-chemical treatments. These active molecules were transformed to exhibit nonspecific activity by such treatments. This nonspecific activity was relatively stable, since its activity was maintained for more than one year in the cold. The nonspecific activity was resistant against dialysis, heat-labile, and dependent on divalent cations, similar to the specific activity. Furthermore, the nonspecific activity was mainly found in the fractions of 20–40% saturation by ammonium sulfate fractionation, and the activity was also found in the fractions eluted with void volume in Sephadex G75 gel filtration. These results suggest the activity is carried by a large molecule substance. Additionally, the activity was not affected by trypsin, protease and neuraminidase. At present, the essence of factor(s) inducing NFR is obscure.

In conclusion, the recognition mechanism of self colony from non-self colony in ascidian is responsible for the interaction between blood cells and humoral factor(s) in the plasma. The humoral factor, or recognition molecule may be generated by lymphocytes. The similarity of the ascidian recognition molecule formation to the antibody formation in the vertebrates is indicated. The analysis on the structure of humoral factor is to be great importance in the consideration of the ascidian recognition mechanism.

REFERENCES

1 Hildemann, W. H. (1972) In "Transplantation Antigens". Ed. by B. D. Kahan and R. A. Reisfeld, Academic Press, New York, pp. 3–73.
2 Hildemann, W. H. (1974) Nature, **250**: 116–120.
3 Hildemann, W. H. and Reddy, L. (1973) Fed. Proc., **32**: 2188–2194.
4 Berrill, N. J. (1955) The Origin of Vertebrates, Oxford University Press, London.
5 George, W. C. (1939) Q. J. Microsc. Sci., **81**: 391–428.
6 Sabbadin, A. (1955) Arch. Ital. Anat. Embriol., **60**: 33–67.
7 Overton, J. (1966) J. Morphol., **119**: 305–326.
8 Goodbody, I. (1974) The Physiology of Ascidians. In "Advances in Marine Biology". Ed. by F. S. Russell and M. Yonge, vol. 12, Academic Press, New York, pp. 1–149.
9 Ratcliffe, N. A. and Rowley, A. F. (1979) Dev. Comp. Immunol., **3**: 189–243.
10 Wright, R. K. (1981) Urochordates. In "In-

vertebrate Blood Cells". Ed. by N. A. Ratcliffe and A. F. Rowley, vol. 2, Academic Press, New York, pp. 565–629.
11 Freeman, G. (1964) J. Exp. Zool., **156**: 157–184.
12 Taneda, Y. and Watanabe, H. (1982) Dev. Comp. Immunol., **6**: 665–673.
13 Warr, G. W., Decker, J. M., Mandel, T. E., DeLuca, D., Hudson, R. and Marchalonis, J. J. (1977) Aust. J. Exp. Biol. Med. Sci., **55**: 151–164.
14 Cantacuzene, J. (1913) C. R. Soc. Biol., **74**: 111–112.
15 Tyler, A. (1946) Biol. Bull., **90**: 213–219.
16 Wright, R. K. and Cooper, E. L. (1975) Am. Zool., **15**: 21–27.
17 Wright, R. K. (1974) J. Invertebr. Pathol., **24**: 29–36.
18 Parrinello, N. and Canicattì, C. (1982) Dev. Comp. Immunol., **6**: 53–64.
19 Coombe, D. R., Schluter, S. F., Ey, P. L. and Jenkin, C. R. (1982) Dev. Comp. Immunol., **6**: 65–74.
20 Fuke, M. T. and Sugai, T. (1982) Biol. Bull., **143**: 140–149.
21 Anderson, R. S. and Good, R. A. (1975) Biol. Bull., **148**: 357–369.
22 Bretting, H. and Renwrantz, L. (1973) Z. Immun-Forsch., **145**: 242–249.
23 Parrinello, N. and Canicattì, C. (1983) Biol. Bull., **164**: 124–135.
24 Coombe, D. R., Ey, P. J. and Jenkin, C. R. (1984) Q. Rev. Biol., **59**: 231–255.
25 Anderson, R. S. (1971) Biol. Bull., **141**: 91–98.
26 Fuke, M. T. (1979) Bull. Mar. Biol. Stn. Asamushi, **16**: 142–159.
27 Bresciana, J. and Lützen, J. (1960) Cac. Biol. Mar., **1**: 157–184.
28 Monnit, C. (1963) Vie Milieu, **14**: 263–273.
29 Dudley, T. L. (1968) J. Morphol., **124**: 263–281.
30 Smith, M. J. (1970) Biol. Bull., **138**: 354–378.
31 Parrinello, N., Patricolo, E. and Canicattì, C. (1984) Biol. Bull., **167**: 229–237.
32 Parrinello, N. and Patricolo, E. (1984) Biol. Bull., **167**: 238–250.
33 Fuke, M. T. (1980) Biol. Bull., **158**: 304–315.
34 Fuke, M. T. and Numakunai, T. (1982) Dev. Comp. Immunol., **6**: 253–261.
35 Bancroft, F. W. (1903) Proc. Calif. Acad. Sci. (3), **3**: 137–186.
36 Oka, H. and Usui, M. (1944) Sci. Rep. Tokyo Bunrika Daigaku, B, **7**: 23–53.
37 Watanabe, H. and Taneda, Y. (1982) Am. Zool., **22**: 775–782.
38 Freeman, G. (1970) Transplant. Proc., **2**: 236–239.
39 Mukai, H. and Watanabe, H. (1974) Biol. Bull., **147**: 411–421.
40 Koyama, H. and Watanabe, H. (1981) Annot. Zool. Japon., **54**: 30–41.
41 Koyama, H. and Watanabe, H. (1982) Biol. Bull., **162**: 171–181.
42 Saito, Y. and Watanabe, H. (1982) Proc. Jpn. Acad., **58**: 105–108.
43 Saito, Y. and Watanabe, H. (1976) Zool. Mag., **85**: 339.
44 Katow, H. and Watanabe, H. (1980) Dev. Biol., **76**: 1–14.
45 Schmidt, G. H. (1982) Biol. Bull., **162**: 195–201.
46 Tanaka, K. and Watanabe, H. (1973) Cell. Immunol., **7**: 410–426.
47 Taneda, Y. and Watanabe, H. (1982) Dev. Comp. Immunol., **6**: 43–52.
48 Saito, Y. and Watanabe, H. (1978) Zool. Mag., **87**: 316.
49 Milanesi, C., Burighel, P., Zaniolo, G. and Sabbadin, A. (1978) Boll. Zool., **45**: 83–86.
50 Oka, H. and Watanabe, H. (1957) Proc. Jpn. Acad., **33**: 657–659.
51 Oka, H. and Watanabe, H. (1960) Bull. Mar. Biol. Stn. Asamushi, **10**: 153–155.
52 Oka, H. and Watanabe, H. (1967) Kagaku (Tokyo), **37**: 307–313.
53 Oka, H. (1970) In "Profiles of Japanese Science and Scientists". Ed. by H. Yukawa, Kodansha, Tokyo, pp. 195–206.
54 Mukai, H. and Watanabe, H. (1975) Proc. Jpn. Acad., **51**: 44–47.
55 Scofield, V. L., Schlumpberger, J. M., West, L. A. and Weisman, I. L. (1982) Nature, **295**: 499–502.
56 Scofield, V. L., Schlumpberger, J. M. and Weisman, I. L. (1982) Am. Zool., **22**: 783–794.
57 Mukai, H. (1967) Sci. Rep. Tokyo Kyoiku Daigaku, B, **13**: 51–73.
58 Tanaka, K. (1973) Cell. Immunol., **7**: 427–443.
59 Taneda, Y. and Watanabe, H. (1982) Dev. Comp. Immunol., **6**: 243–252.
60 Taneda, Y. (1985) Dev. Comp. Immunol. (in press).
61 Katow, H. and Watanabe, H. (1974) Zool. Mag., **83**: 146–151.
62 Saito, Y. and Watanabe, H. (1983) Zool. Mag., **92**: 498.
63 Saito, Y. and Watanabe, H. (1984) Zool. Sci., **1**: 229–235.