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Self or Non-self Recognition in Compound Ascidians[1]

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SYNOPSIS. Certain species of compound ascidians have an ability to distinguish self colonies from non-self colonies within the same species. This ability, called colony specificity, is manifested by the fusibility between colonies. The fusibility among colonies of Japanese *Botryllus* is genetically controlled by a series of multiple alleles at a single locus. The fusibility is determined by a factor(s) in blood, so that the fusibility can be altered by the exchange of blood. It is suggested that rejection, called "nonfusion" reaction, may occur from the interaction between blood cells and blood humoral factor(s).

INTRODUCTION

Among the vertebrates, even the most primitive living forms, the Agnathans, show an antibody response and reject foreign grafts (Hildemann, 1970). There is no report that any invertebrate synthesizes specific antibodies, though many immune-like responses have been known among diverse invertebrates. The origins of immunological reactivity are suggested to lie within the invertebrates (Hildemann and Cooper, 1970; Hildemann, 1972).

In certain species of compound ascidians, a phenomenon known as colony specificity exists. This was first noted by Bancroft (1903) in *Botryllus*. These animals belong to the phylum Protochordata. Since it is generally believed that the vertebrates evolved directly from this group (Berrill, 1955), colony specificity in compound ascidians should offer some insight into the origins of the vertebrate immune responses.

NATURE OF COLONY SPECIFICITY

Occurrence of colony specificity

When two colonies belonging to the same species make contact with their growing edges or cut surfaces, they either fuse with each other to form a single colony in one case or they do not fuse. 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 "nonfusion" reaction (NFR), in other words "rejection."

In other species, *e.g., Polycitor mutabilis*, a different phenomenon is observed at the contact area between the growing edges. Neither the complete union of the test matrix nor the particular rejection between two colonies was observed at the contact area, regardless of their origin. This phenomenon was designated as "indifference."

In *Botrylloides simodensis* (Saito *et al.*, 1981), "fusion" or "rejection" was demonstrated at the cut surfaces, whereas "fusion" or "indifference" occurred at the growing edges (Tanaka and Watanabe, 1973; Mukai and Watanabe, 1974). Recently in this species "rejection" was observed at the

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TABLE 1. *Summary of the occurrence of colony specificity in Japanese compound ascidians.*

Species	Contact with		References
	Growing edges	Cut surfaces	
Botryllus primigenus	F or R	F or R	Tanaka and Watanabe, 1973; Mukai and Watanabe, 1974
Botryllus scalaris n. sp.	F or R	F or R	Saito and Watanabe, 1978
Botrylloides simodensis	F or R*	F or R	Saito and Watanabe, 1976
Symplegma reptans	F or R	F or R	Mukai and Watanabe, 1974
Didemnum moseleyi	F or R	F or R	Mukai and Watanabe, 1974
Perophora orientalis	I	F	Mukai and Watanabe, 1974
Perophora japonica	F or R	–	Koyama and Watanabe, 1981
Polycitor mutabilis	I	F	Oka and Usui, 1944
Aplidium yamazii	F or R	F or R	

* partial rejection: F, "Fusion"; R, "Rejection"; I, "Indifference"; –, No experiment was performed.

growing edges, though there was partial fusion (Saito and Watanabe, 1976).

In other genera for which no published reports of "rejection" have appeared yet, "rejection" at the growing edges has been discovered recently by a Japanese group. Therefore, "indifference" must be more carefully considered.

The results of fusion experiments by our group are summarized in Table 1.

Genetic control of fusibility in Botryllus primigenus

Oka and Watanabe (1957, 1960) investigated the fusibility between the colonies taken from nature and among F1 or F2 progenies derived by the crossing of two nonfusible colonies (in *B. primigenus* self-fertilization does not occur). The results obtained can be summarized as follows: (a) Almost all colonies taken at random from nature do not fuse with each other. (b) F1 colonies are sorted into four groups, which appear roughly in proportions of 1:1:1:1. Members of each group fuse with one another and with those of two other groups. (c) Fusion is always possible between daughter colonies and the mother colony. (d) F2 colonies obtained by crossing of two nonfusible F1 colonies are also sorted into four groups in the same manner as in F1. From these results they postulated the following hypothesis: (1) Each colony in nature is heterozygotic with respect to the gene governing fusibility. (2) The gene is represented by a series of alleles like the S gene governing self-incompatibility in flowering plants. (3) Colonies containing at least one allele in common are fusible with one another, while those containing no common allele are nonfusible. A schematic illustration of this hypothesis appears in Figure 1.

In order to examine both the validity of these ideas and the actual distribution pattern of alleles controlling fusibility in *Botryllus primigenus,* fusion experiments were carried out on a large number of colonies collected from three areas of the Izu peninsula (Oka, 1970; Mukai and Watanabe, 1975). No exception to the rules described above was found, thus implying very strongly that fusibility among colonies of this species is in fact controlled by a series of multiple alleles at a single locus. These genes were designated F1, F2, F3, . . . , in the same way as the S genes of higher plants.

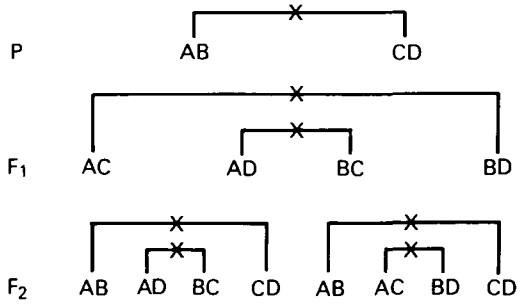


FIG. 1. Schematic diagram of the hypothesis on fusibility of *Botryllus primigenus*. —x—, nonfusible; otherwise fusible.

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. 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 (hemihomologous colony) (Oka and Watanabe, 1957; Mukai, 1967). According to Mukai, a colony BC was fused with a sister colony AC of the same size. Four days after fusion, the two colonies were separated again. Then, BC colony became nonfusible with BD colony which should be originally fusible. In his series I experiments, relative sizes of the two colonies BC and AC were varied. When BC colony was larger than AC colony, acquired fusibility of BC (to become nonfusible with BD) returned to original fusibility after certain periods of time. The smaller the relative size of AC colony to BC colony, the shorter the time required for returning to original fusibility of BC. In the same manner, AC colony became fusible with BD colony by fusion and re-separation of BC colony. The larger the relative size 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.

Feature of "nonfusion" reaction (NFR)

When two incompatible colonies of *B. primigenus* came into contact with the growing edges, rejection, called "nonfusion" reaction (NFR), took place at the contact area. The process of NFR is schematically illustrated in Figure 2. This reaction proceeded as follows. The tips of the ampullae surrounding the periphery of each colony actively extended toward the other, and first the margins of test matrices of two colonies contacted each other (stage 1). One or two hours after the contact, the tips of the ampullae pushed against each other and penetrated into the test matrix of the opposite colony (stage 2). No change was observed in the contact area at this stage. Two or three hours later, the penetration of ampullae into the test matrix was further advanced, and the first sign of NFR was recognized (stage 3) by the increase in opacity at the tips of the ampullae. We discovered the infiltration of morula cells in this stage. If India ink was injected into the blood vessels prior to the occurrence of NFR, it leaked from the tips of ampullae of the contact area into the test matrix at this stage. Therefore, an increase in the permeability of the ampullar epidermis was suggested.

Seven hours after contact, the ampullae of both colonies stopped further penetration into the test matrices of the opposite colonies (stage 4). Two or three hours after reaching stage 4, the ampullae began to contract and to become thinner at their proximal parts, where blood flow decreased (stage 5). Two hours after the preceding stage, the contraction of the ampullae further proceeded, and the blood stream in the ampullae stopped completely. Finally, the distal parts of the ampullae were constricted off from the healthy parts of the proximal vascular system, and new walls were formed to separate the degenerated zone from the healthy parts of both colonies facing each other (stage 6) (Tanaka and Watanabe, 1973; Taneda and Watanabe, 1982).

Electron microscopic 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 even at stage 6 of NFR (Tanaka and Watanabe, 1973).

ANALYSIS OF THE MECHANISM OF NFR

Where does the first step of NFR take place?

From light and electron microscopic observations, Tanaka and Watanabe (1973) tentatively interpreted that the first step of NFR took place in the test cells in the test matrix. When either one of two incompatible colonies was experimentally removed at stage 3 of NFR, NFR proceeded irreversibly in the other colony remaining on the glass slide at the same rate as NFR ob-

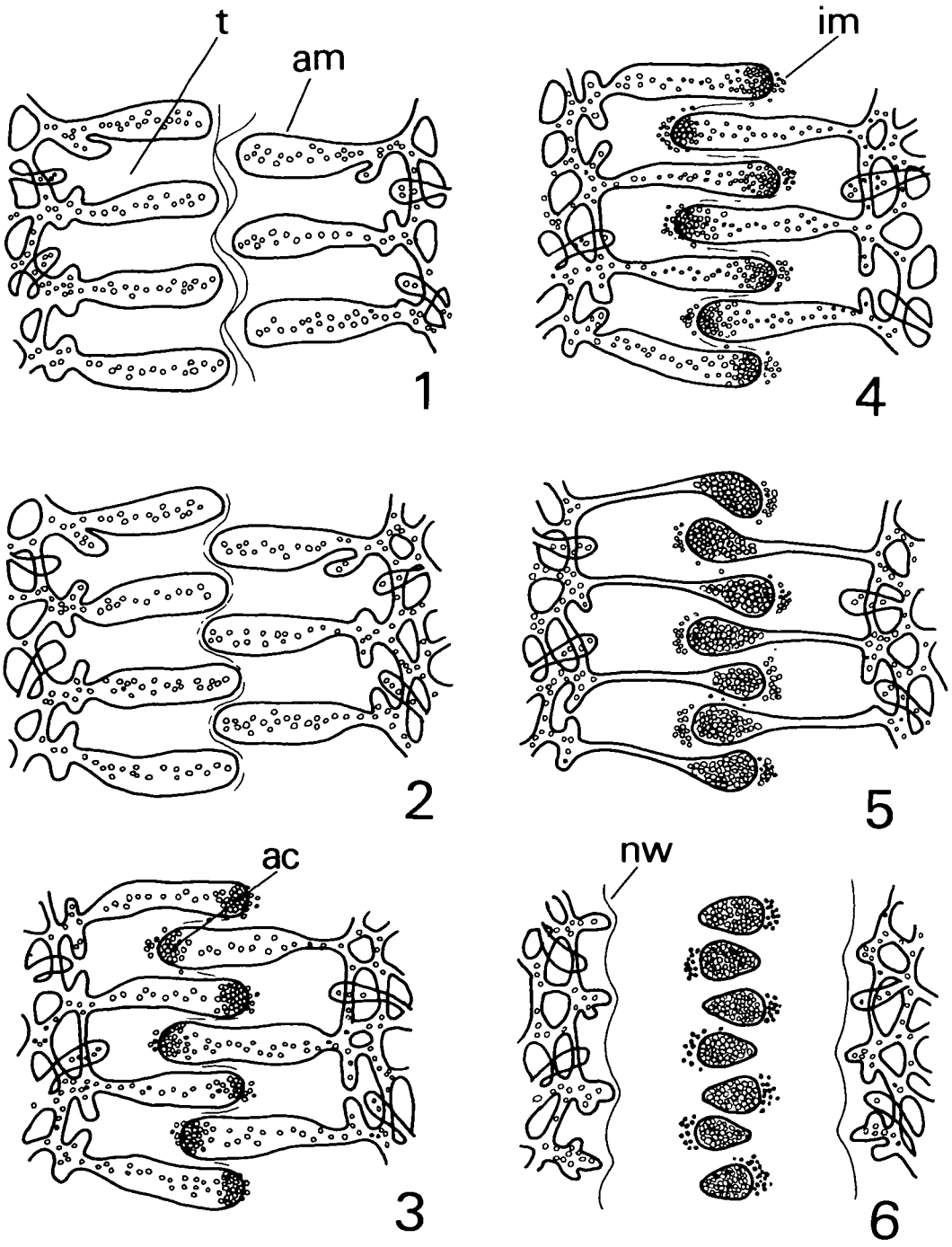


FIG. 2. Schematic illustration of the process of "nonfusion" reaction (NFR). Numbers indicate stages of NFR. See text for details. ac, aggregate of blood cells; am, ampulla; im, infiltrated morula cells; nw, new wall; t, test matrix.

served in colonies which were not disturbed (Tanaka, 1973; Fig. 3a).

In order to determine whether the first step of NFR takes place in the test matrix or in the ampullar lumen, we examined whether or not a colony piece previously contacted with an incompatible colony until stage 3 of NFR could induce NFR to another compatible colony. On the basis of our results, it appeared that the first step of NFR took place in the ampullar lumen (Fig. 3b). The results of other experiments, that is, the contact of a colony piece with an incompatible zooid-free ampullae fragment or overlapping of one colony with another incompatible colony, suggested that only when the ampullae penetrated into the test matrix of the opposite colony did NFR take place in the penetrated ampullae (Fig. 3c, d).

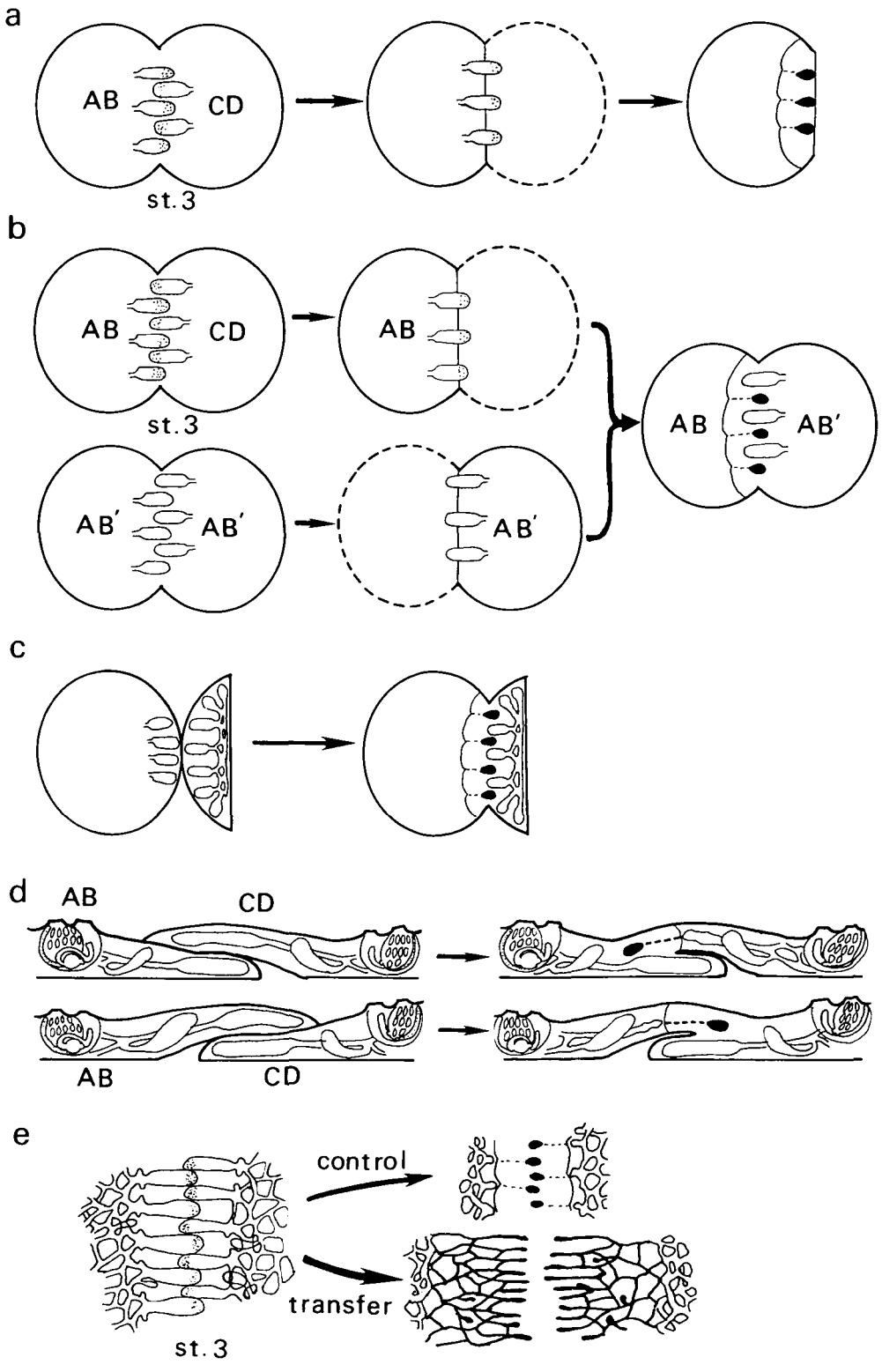
Finally, if the first step of NFR really takes place in the ampullar lumen, the "reacting" blood in the ampullar lumen may induce NFR at other vessels which are in healthy condition. From this point of view, we attempted to transfer the "reacting" blood to other healthy parts in the vessels by pushing gently with a pair of forceps. The stoppage of blood flow and subsequent constriction of vessels always appeared in the transfer area (Fig. 3e). The results of this experiment also suggested that the first step of NFR took place in the ampullar lumen.

Mechanism of NFR

Tanaka (1973) performed suggestive experiments on the mechanism of NFR. A piece was taken from each of three strains of F1 colonies, AC, BC, and BD. The pieces were placed side by side on a glass slide in the following order, AC–BC–BD, with the growing edges of the colonies facing each other at proper distance. AC and BD colonies are not fusible, so the blood of the two colonies cannot be mixed in the natural way. However, when a BC colony was placed between an AC and BD colony, the blood of AC and BD were mixed within the vessels of the BC colony after the establishment of connections on both sides of the BC colony. When the fusion at both sides occurred almost at the same time, the formation of clusters of blood cells (aggregation or agglutination?) appeared within the vessels at the middle region of the BC colony and at the contact regions of both sides within a few hours. Subsequently, the vessels in the three regions gradually constricted to stop the blood flow and these regions finally necrosed (Fig. 4). Electron microscopic observations revealed the formation of filaments in intracellular spaces of the blood cell cluster (Tanaka, 1973). The results obtained from this experiment suggested that blood contained both an "effector agent" and a "recognition agent" concerning NFR, and that NFR took place by the mixture of blood from two incompatible colonies.

Thus, we attempted to inject the blood obtained from an incompatible colony into the ampullae of the recipient. No response was induced by the injection of blood into the same colony. On the other hand, the injection of blood into the incompatible colony resulted in remarkable responses. Such responses were characterized by formation of clusters of blood cells within the vessels, a contraction of the vessels and ampullae, and subsequent regression of the zooids. In the most remarkable specimens, the infiltration of morula cells constricting off the vessels were observed. These responses were similar to NFR. Moreover, we challenged two fractions of blood, blood plasma and blood cells, respectively. The challenge of blood cells into the incompatible colony resulted in remarkable responses which were the same as whole blood injection. A weak response was induced by the injection of blood plasma into the incompatible colony. The results of injection of whole blood or blood fractions are summarized in Table 2. Injection of blood suggested that the cluster formation, or the first step of NFR, was initiated by the interaction of blood cells and blood humoral factor.

From these results we consider the mechanism of NFR to be as follows: A humoral factor(s) concerning NFR exists in the test matrix as well as in the blood. When two incompatible colonies come into con-



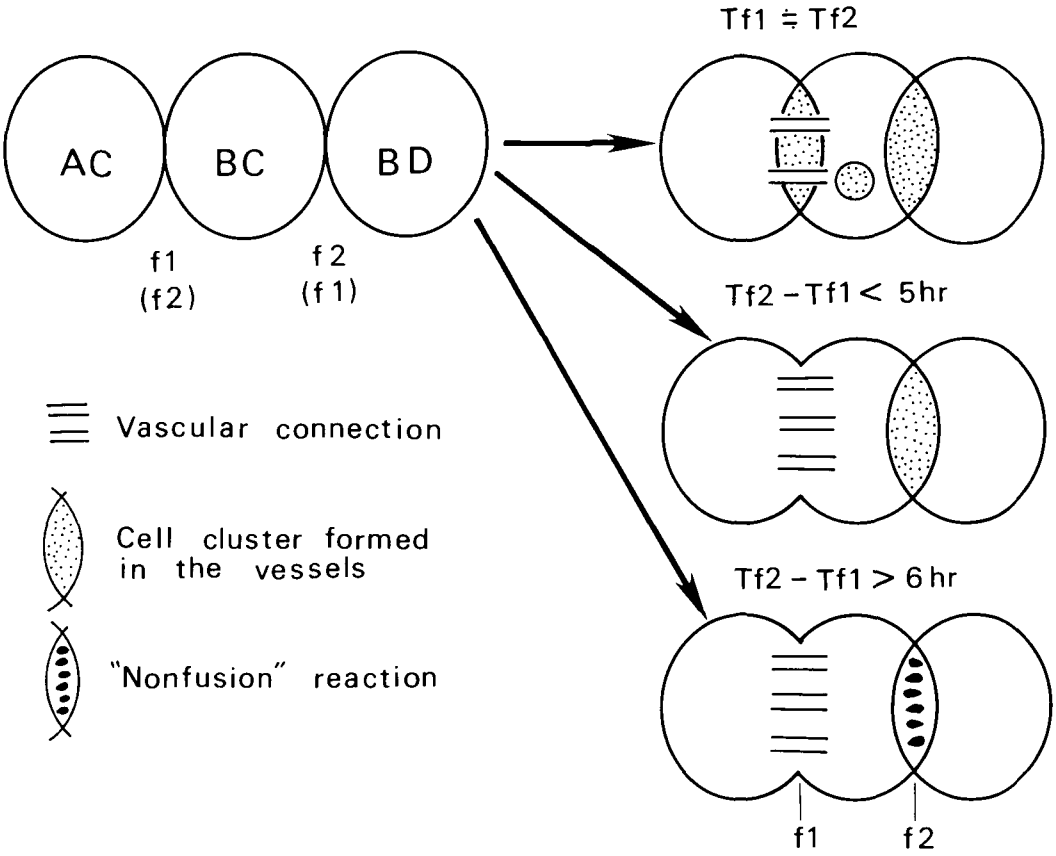


FIG. 4. Schematic illustration of the fusion experiments using three F1 colonies. f1, earlier fusion; f2, later fusion; Tf1, time of earlier fusion; Tf2, time of later fusion (from Tanaka, 1973). See text for details.

tact and the ampullae penetrate into the test matrix of opposite colony, uptake of this factor into the lumen of the penetrating ampullae takes place. This factor reacts only with non-self blood cells and the formation of a cluster of blood cells occurs 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 of blood flow and the constriction of the vessels. The destruction of test cells and the formation of new wall occurs subsequently.

Nature of the factor(s) concerning NFR

By the use of Mukai's fusibility alteration technique (Mukai, 1967), we revealed that a factor(s) concerning fusibility was sensitive to X-irradiation. This result suggested that at least one factor concerning fusibility might be a cellular component in blood, supporting the conclusion obtained from

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FIG. 3. Schematic illustration of the experiments on the mechanism of NFR. (a) Removing of one colony at stage 3 of NFR resulted in NFR at the same rate as natural NFR. (b) One colony which was in contact with an incompatible colony until stage 3 of NFR did not induce NFR in another compatible colony. (c) Contact between a colony piece and an incompatible zooid-free ampulla fragment resulted in NFR only at the colony piece. (d) Overlapping of one colony on another resulted in NFR only in the "upper-colony." (e) Transfer of the "reacting" blood from the opaque areas of the ampullae to the healthy parts of the vessels induced NFR in the healthy parts of the vessels.

TABLE 2. *Injection of whole blood or blood fractions derived from syngeneic or allogeneic colonies, respectively.*

Injected materials	Total number of challenges	Results ++	+	−
1. Syngeneic blood				
a) Whole blood	51	0	8	43
b) Blood plasma	42	0	3	39
c) Blood cells	37	0	3	34
2. Allogeneic blood				
a) Whole blood	33	31	1	1
b) Blood plasma	56	5	44	7
c) Blood cells	40	35	4	1

++, strong positive response; +, weak positive response; −, no response.

the injection of blood fractions. Since lymphocyte population was decreased in the irradiated preparations in which a factor concerning fusibility was also reduced, these cells may play a role in colony specificity.

The nature of the humoral factor concerning fusibility is not known. Several authors reported that some solitary ascidians possessed hemagglutinin in the blood (Fuke and Sugai, 1972; Anderson and Good, 1975; Wright and Cooper, 1975). Such hemagglutinins may be the most likely candidates for the humoral factor concerning fusibility in *Botryllus*. We are now studying this possibility by the use of the *in vivo* bioassay technique based on the micro-injection of blood.

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