Table 1. Invagination and evagination of transposed wing grafts. In scoring configurational changes of grafts, all transposed epithelia that occupied a position below the plane of the surrounding host epithelia were considered to be invaginated and those above to be evaginated. Variations occurred, from slight graft inpocketings and outpocketings (Fig. 2, D to G) to complete segregation of graft from host tissue. In the latter, a vesicle either formed between upper and lower epithelial layers of the wing (invagination) or was found lying free on the apical surface of the upper epithelial layer of the host (evagination).

Graft-host combination	Number of exchanges	Percentage of grafts (0p and Ip) that invaginated	Percentage of grafts (VIIa and Vp) that evaginated
	2 by 2 mm graft (4×10^4 cells)		
Ip ↔ VIIa	28	7	43
Ip ↔ Vp	26	4	38
0p ↔ VIIa	14	14	43
0p ↔ Vp	15	20	87
	1 by 1 mm graft (1×10^4 cells)		
Ip ↔ VIIa	16	56	81
Ip ↔ Vp	18	58	83
0p ↔ VIIa	12	100	100
0p ↔Vp	11	100	100

grafts moved to distal positions may simply be attributable to their large size; further decreases in size of transposed grafts should be accompanied by an increased frequency of invagination. When the apical surface area of the graft is reduced by one-quarter, the frequency of invagination is markedly increased (Table 1 and Fig. 2, D and E).

The frequency of invagination should be further accentuated by transposition of tissue from the more proximal region 0p to VIIa and Vp, since adhesive disparity between cell populations is intensified as the distance separating them along a proximodistal adhesiveness gradient increases (Table 1 and Fig. 2G).

The otic placode of the chick occupies an area of 60,000 μm^2; other embryonic placodes are comparable in size (*9*). Since cellular dimensions for wing epithelial cells match those for cells of vertebrate epithelia, we can estimate that hundreds, rather than thousands, of cells are usually involved in invagination. Below a certain size, all distally transposed wing grafts from region Ip may invaginate (Table 1). A corollary claim would be that adhesive disparity between placode and surrounding epithelium should increase as dimensions of an invaginating region increase.

Microtubules and microfilaments are generally accepted as the agents responsible for changes in cellular form during invagination (*1, 10*). The contention that cytoskeletal elements are the primary forces behind folding movements has been based mainly on the use of drugs that, although they disrupt microtubules and microfilaments, also affect the cell surface (*7*). Even though all cells in wing epithelia of pupal and developing adult moths are richly endowed with microtubules oriented along the apicobasal axis of the cell and with microfilaments circumscribing the apex of the cell (*11*), these cells normally do not invaginate or evaginate. Microtubules and microfilaments may simply stabilize forms adopted by cells under the influence of other cellular forces (*12*). These forces could be generated by adhesive properties of cell surfaces as cells adopt configurations of minimum free energy (*8*).

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The Complement System of the Nurse Shark: Hemolytic and Comparative Characteristics

Abstract. *The complement system of the nurse shark was investigated. Six functionally pure components were isolated from a single serum sample. Sequential reactions of the components with sensitized sheep erythrocytes resulted in membrane lesions indistinguishable from the "holes" caused by guinea pig complement.*

The nurse shark, *Ginglymostoma cirratum*, has survived 350 million years of evolution. We have separated from the serum of this ancient species six functionally pure complement components (*1*); their sequential interactions result in the lysis of sensitized target cells. We present evidence for the existence of this six-component system and for the classical pathway (*2*) of its activation, which results in membrane lesions that are indistinguishable from the "holes" produced by mammalian complement.

Naming components of a complement (C) system, shorter than the nine-component mammalian one, was problematic because numbers or letters imply analogies that may not be appropriate. In the past, we used "n" to indicate nurse shark origin; thus, nurse shark antibody was designated An and sheep erythrocytes sensitized with that antibody were designated EAn. In 1973 we isolated, purified, and characterized the first component of the system and called it C1n (*3*).

Subsequently, we found that the mammalian intermediate complex, EAC1–7, could be lysed by two individually inactive serum fractions which we assumed to be the preterminal and terminal components of the shark system. After their isolation and purification we called them accordingly t1 and t2 (*4*). Three additional components, X, Y, and Z, discovered more recently, reacted after C1n and before t1. The reaction sequence of the six components was C1n-

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Y-X-Z-t1-t2. No mammalian components could be substituted for Y, X, or Z and none of the three would react with any mammalian intermediate complexes. Following a suggestion by Dr. Hans Müller-Eberhard, we adopted a compromise numbering system; in recognition of the analogies with the corresponding mammalian components, the first component remained C1n, and t1 and t2 were renamed C8n and C9n, respectively. The components Y, X, and Z became C2n, C3n, and C4n, reflecting their order in the overall reaction sequence: EAn-C1n-C2n-C3n-C4n-C8n-C9n.

The following is a brief description of the procedures by which the six components can be obtained from a single serum sample (*5*). Dilution of chilled shark serum with ice-cold distilled water to achieve a conductance of 4 mS at *p*H 7.4 yields a precipitate that contains more than 95 percent of the C1n. The method of purification and the functional and physicochemical characteristics of this component have been described (*3*). The supernatant from the low ionic strength precipitation contains the other components (*6*). The supernatant can be used directly as starting material for anionic ion-exchange chromatography; if it is applied to a DE-52 column at 4 mS and *p*H 7.4, all components are retained except C8n, which is recovered from the effluent. Upon elution with a salt gradient, C9n peaks between 9 and 10 mS, and C4n peaks at 12 mS. Since C3n elutes only slightly later than C4n, these two components cannot be separated on DE-52; C2n elutes at 15 to 16 mS. Preparations of C8n, C9n, and C2n that are functionally, though not chemically, pure (*1*) can usually be obtained by rechromatography of pools from the peak fractions. Hydroxyapatite column chromatography with a phosphate gradient can be used for separating C3n and C4n. At *p*H 6.8 and a salt concentration of 0.15*M* NaCl, C3n elutes at 0.05*M* phosphate, and C4n elutes between 0.2*M* and 0.25*M* phosphate. Hydroxyapatite chromatography is also useful for further purification of C8n and C9n, which elute at 0.2*M* and 0.05*M* phosphate, respectively. Highly purified C9n has a molecular size of 185,000 daltons (*4*). As judged by gel filtration, C8n is slightly larger than C9n, and all other components appear to exceed 200,000 daltons.

Stepwise formation of intermediate complexes was used for the assessment of reaction conditions and the titration of individual components. The complexes were prepared by adding to one volume of cells (1×10^8 cells per milliliter) an equal volume of the component dilution containing about 50 units of 50 percent hemolytic complement (CH_{50}) per milliliter. The mixture was then incubated (with shaking) in a water bath, usually for 30 minutes at the indicated temperature. The reaction was stopped by addition of five volumes of cold buffer and immediate centrifugation; the cells were then washed twice in cold buffer. The buffer was always $DGVB^{2+}$ (*1*). Whether or not complex formation had occurred under various conditions was determined by using the cells for titrations with the next component (Table 1). The formation of the first complex, EAnC1n, requires shark antibody and proceeds in the presence of 0.04*M* EDTA since C1n is more resistant to this chelating agent than mammalian C1 (*3*). The next complex, EAnC1nC2n, forms readily at 30°C but slowly at 0°C; it is stable and can be kept reactive for several days at 0°C. The third complex, EAnC1nC2nC3n, forms at 0°C and is unstable at 30°C. Formation of the fourth complex, EAnC1nC2nC3nC4n, proceeds at 30°C; it is stable and remains reactive for several days if kept at 0°C. The second, third, and fourth intermediate complexes do not form in the presence of 0.02*M* EDTA. The stability of the fourth complex and its effective interaction with mammalian C8 and C9 is reminiscent of the mammalian intermediate complex EAC1–7. Both form a new complex with the next component, which is then lysed rapidly and efficiently by the last.

Whether the erythrocyte is lysed by whole shark serum containing natural antibodies to E together with all complement components or whether it is lysed by stepwise exposure to antibody and purified individual complement components, the membrane lesions are the same. Those shown in Fig. 1 were obtained by treating E sequentially with natural antibody and the six complement components.

With an average diameter of 80 Å and identical morphology, the lesions are indistinguishable from those caused by guinea pig complement; they differ only in size from lesions caused by human complement, which range from 100 to 110 Å (*7*).

Until all components can be obtained in chemically pure form it might be argued that additional activities could be hidden in one or more of the identified components. We are reasonably certain that this is not the case because (i) we have consistently observed that the specific activity of all components increases with the degree of purification; (ii) each component shows distinct elution characteristics on column chromatography; (iii) each component alone is necessary and sufficient for the formation of its corresponding intermediate complex; (iv) each component has been obtained in functionally pure form (see legend to Table 1); and (v) all components except C1n can be recovered at distinct areas from polyacrylamide gel after electrophoresis (7.5 percent gel; *p*H 8.9). Under these conditions a mixture of C2n, C3n, and C4n subjected to electrophoresis showed relative mobility (R_F) values of 0.23, 0.20, and 0.07, respectively.

With few exceptions, components of mammalian C systems can be freely exchanged. The species compatibilities

Table 1. Methods used for the hemolytic measurements of the individual components of nurse shark complement. In all cases a total of five volumes is included in each reaction mixture. The volumes are 0.5 ml for tube titrations and 0.25 ml for microtiter titrations; 50 percent hemolytic endpoints are calculated or estimated, respectively. Cells and reagents are added in the order shown. Volumes of diluent ($DGVB^{2+}$) are added if necessary, with the last step. The reagent components contained 50 to 100 CH_{50} units per milliliter. Controls, consisting of cells, reagents, and diluent, must show no lysis, thus indicating functional purity of the reagents.

Component dilutions	Intermediate complex	Reagent	Temperature (°C)	Time (minutes)
C1n	EAn		30	20
		Guinea pig supertatant I (1:8)*	30	60
C2n	EAnC1n		30	20
		C3n, C4n	30	20†
C3n	EAnC1nC2n		30	20
		C4n	30	20†
C4n	EAnC1nC2nC3n or		30	20†
	EAnC1nC2n + C3n		30	20†
C8n	EAC1gp4–7hu or	C9n	37	60
	EAnC1n–4n	C9n	30	60
C9n	EAC1gp4–7hu or	C8n	37	60
	EAC1gp–7hu–8gp or		37	60
	EAC1n–4n	C8n	30	60

*See (*3*) †Followed by C8n, C9n, 30°C, 60 minutes.

that allow this exchange are considered to reflect molecular similarities among the interchangeable components. Even certain incompatibilities, if they arise from ineffective complex formation (*8*), may indicate structural relationships. Definitive phylogenetic studies must depend on structural information that is still incomplete even for the components of the human and guinea pig systems, which have been intensively investigated for many years. The C systems of most other mammalian and of all premammalian species still need to be studied. Components need to be isolated and separated. Reaction sequences and conditions for the formation of intermediate complexes must be established. Only then can C components and systems be judiciously compared, compatibility studies conducted, and developmental relationships investigated.

As expected, several absolute and partial incompatibilities were apparent when we tried to exchange mammalian and shark components. We were surprised, however, to find similarities and compatibilities, clearly indicating preservation of distinct component functions. Shark antibody is incompatible with human, guinea pig, and dog C1, but is compatible with rabbit C1. Shark C1n is incompatible with rabbit antibody (IgG or IgM), but reacts readily with dog IgM. Although EAnC1n reacts with guinea pig or human C4 and C2 (*3*), mammalian EAC1 is incompatible with shark component C2n. The stable mammalian complex EAC1–7 is compatible with C8n, but the resulting complex, which is readily lysed by C9n, is incompatible with human and guinea pig C9. Shark C9n is compatible with guinea pig C8, but not with human C8. The shark intermediate complex EAnC1n–4n can be lysed by mammalian C8 and C9, just as the corresponding mammalian complex is lysed by the two terminal shark components. In both instances, however, lysis by the heterologous components is about 20 times less efficient than lysis by the homologous ones.

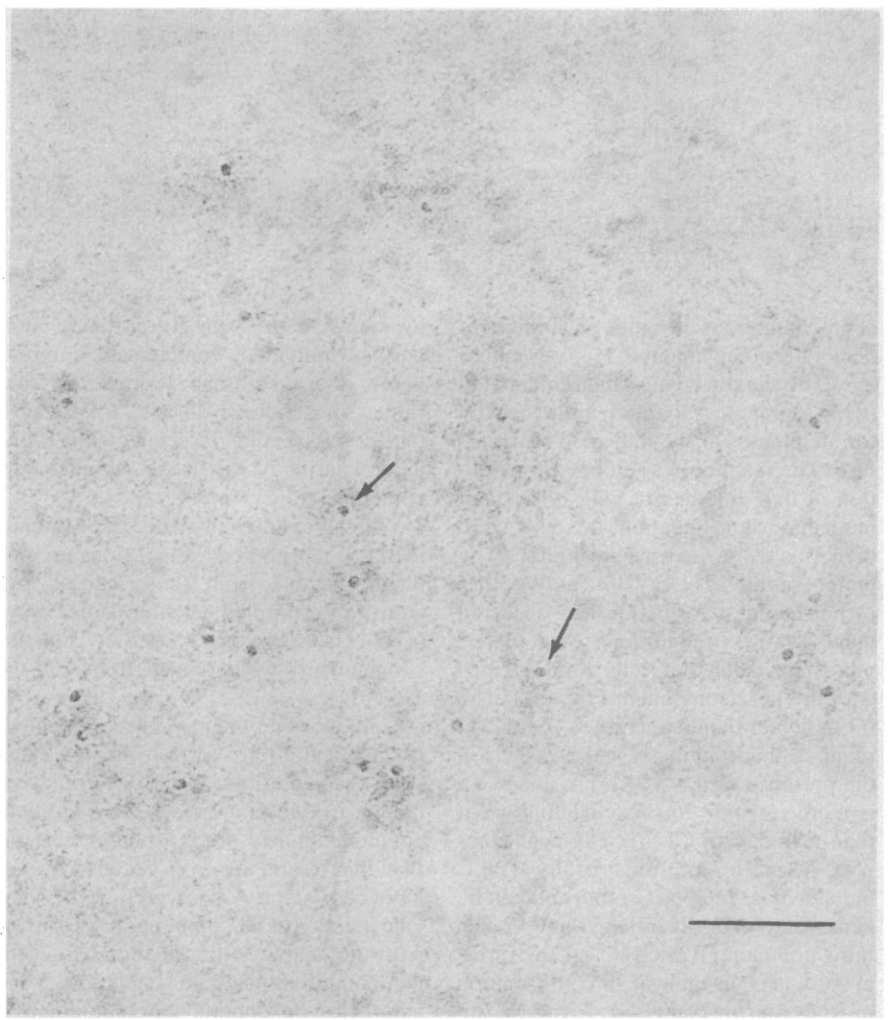

Fig. 1. Sheep erythrocyte stroma showing lesions (arrows) caused by nurse shark complement. Sheep erythrocytes were optimally sensitized with natural nurse shark antibody and reacted with C1n (*3*). The cells were washed three times in cold $DGVB^{2+}$ and suspended in the same buffer at 5×10^8 cells per milliliter; 0.5 ml of the cell suspension was then mixed with 0.5 ml of purified C2n, incubated in a shaking water bath at 30°C for 30 minutes, washed three times in cold $DGVB^{2+}$ and again suspended in 0.5 ml of the same buffer. The same procedure was repeated with C3n and C4n. The purified components used for each step were diluted in $DGVB^{2+}$ so that they contained approximately 500 CH_{50} units per milliliter, as determined by standard titrations with 1×10^8 cells per milliliter (Table 1). The supernatant fluid after each reaction step showed less than 2 percent lysis. To the EAnC1nC2nC3nC4n that was formed during this procedure, was then added 0.5 ml of a mixture of C8n and C9n containing 250 CH_{50} units each. After 60 minutes of incubation at 30°C, 95 percent of the cells were lysed. In control reaction mixtures containing buffer instead of C8n and C9n less than 2 percent of the cells were lysed. The stroma contained in the lysed sample were washed free of hemoglobin by repeated high-speed centrifugation in a Fisher centrifuge at 7000*g* and then suspended in isotonic saline. The red cell ghosts were allowed to settle on collodion carbon-coated grids. After excess fluid was removed, the grids were negatively stained with 1 percent neutralized phosphotungstic acid and examined with an electron microscope (Philips 200). There was an average of 60 lesions per square micrometer. Scale bar, 0.1 μm. Magnification, ×200,000.

Large amounts of highly purified components of nurse shark complement must be obtained before they can be compared structurally with their analogs. Even then, relationships will be difficult to establish because of the pronounced incompatibilities and the difference in the number of components, which may indicate that a sequence of phylogenetically younger components had a single ancestor. In view of the evidence for a common genetic ancestry of mammalian C3 and C5 (*9*) and the analogy between the mammalian intermediate complex EAC1–7 and shark EAnC1n–4n, it seems possible that C4n is the precursor of the four mammalian components, C3, C5, C6, and C7. The mammalian C3 convertase C142 would then correspond to the shark's C1nC2nC3n; rapid decay is characteristic of both.

The serum of unimmunized nurse sharks contains an unusually high concentration of immunoglobulins with natural antibody activity against an imposing number of different natural antigens. These antigens are found on erythrocytes, leukocytes, lymphocytes, tissue, and tumor cells of many species, as well as on bacteria and viruses. Shark natural antibodies exhibit an unparalleled multitude of distinct specificities (*10*) rather than "polyspecific" combining sites (*11*). Fresh, untreated shark serum invariably destroys the antigenic targets and, as in the hemolytic system, lysis and destruction are complement-mediated. It appears that the combination of a

vast repertoire of natural antibodies with a potent complement system provides the shark with a remarkable—and possibly unique—natural humoral immune system. Little is known about the evolution of natural immunity. It seems that phylogenetically younger species must rely increasingly on overt antigenic stimulation.

If the destruction of sensitized foreign structures may be considered the most basic and dramatic function of the complement system, 350 million years of phylogeny have left it nearly unaltered. The more recent development into a nine-component system could be viewed as a refinement, necessitated by a call for more sophisticated phlogistic activities and corresponding safety and control mechanisms. It appears that the classical pathway of complement activation indeed has a remarkable evolutionary survival value.

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References and Notes

1. Abbreviations and definitions: C, complement; C1, C2, . . . , C9, the various components of complement; E, sheep erythrocyte; EA, sensitized E; EAC1–7, EA reacted sequentially with the first seven mammalian C components; $DGVB^{2+}$, isotonic dextrose-gelatin-Veronal buffer containing divalent calcium (0.00015*M*) and magnesium (0.0005*M*); EDTA, ethylenediaminetetraacetate; functionally pure, not contaminated with another C component; sensitized, reacted with specific antibody; intermediate complexes, sensitized E reacted sequentially with various C components, each conferring reactivity for the next component.
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DNA Synthesis in Cultured Adult Cardiocytes

Abstract. *Trypsin-dissociated atrial cardiocytes from adult rats were exposed to [^3H]thymidine for sequential 24-hour periods from day 2 to day 12 of culture. On day 3 and each day thereafter, cells were prepared for ultrastructural radioautography and examined with an electron microscope. Maximal incorporation occurred on day 5, when 63 percent of the cardiocytes were labeled. Mitotic activity was never present in more than 0.5 percent of the cardiocytes examined. Incorporation of [^3H]thymidine and mitosis occurred only in immature cardiocytes characterized by subsarcolemmal primry filaments and Z bands with or without specific granules; more mature cardiocytes were never labeled.*

Long-term cultures of isolated myocardial cells from the ventricles of fetal and neonatal mammals have provided information on the contraction, metabolism, pharmacology, and electrical properties of heart muscle (*1, 2*). These cardiocytes are capable of DNA synthesis and mitosis both in vivo (*3, 4*) and in vitro (*5, 6*). The same is true for atrial cardiocytes from neonatal rats (*7*). Since DNA synthesis and mitosis are restricted in adult mammalian cardiocytes in vivo (*3, 4*), it is desirable to develop techniques for investigating these activities in adult mammalian cardiocytes in vitro. Ventricular cardiocytes isolated from adult mammals have survived only for short periods (*8, 9*) and have not shown mitotic activity (*10*). We now report that atrial cardiocytes from adult rats can be maintained in culture, where they exhibit intense DNA synthesis and moderate mitotic activity.

Atrial cardiocytes from 300- to 350-day-old female Sprague-Dawley rats (Biobreeding Laboratories) were cultured (*7*). The atria were excised from 40 to 50 hearts, placed in Eagle's minimal essential medium (Gibco), washed twice in Hanks solution (Gibco) without calcium and magnesium, and dissociated at 37°C in 0.1 percent trypsin (Difco). The cultures were enriched in cardiocytes by the selective plating technique of Kasten (*2*): the dissociated cells were preplated for three 1-hour periods, followed by one 24-hour period in gelatin-coated 25-cm culture flasks (Corning) to allow attachment of endothelial cells and fibroblasts.

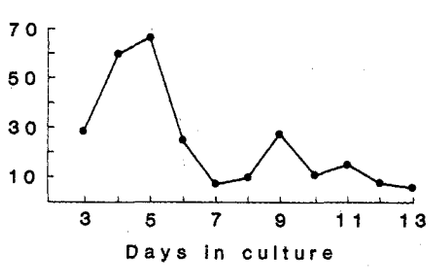

Fig. 1. Percentage of cardiocytes (N = 1000) showing labeled nuclei and subsarcolemmal filaments with forming Z bands or specific granules.

The final supernatant was plated at density of 2×10^6 to 3×10^6 cells in 6 ml of Eagle's minimal essential medium buffered with Hepes at *p*H 7.4 and supplemented with 10 percent fetal calf serum, 1 percent glutamine, and 1 percent penicillin and streptomycin (Gibco). The medium was renewed on days 4, 7, and 10. The cultures were incubated in air at 37°C in gelatin-coated 25-cm flasks.

Cardiocytes started to attach at the end of day 2, grew steadily, and reached confluence on day 8. At this stage they elongated and enlarged to form a criss-cross pattern of branching cells. For sequential 24-hour periods from day 2 to day 12, cardiocytes were exposed to [*methyl*-^{3}H]thymidine (1 μCi/ml; specific activity, 20.0 Ci/mmole; New England Nuclear) (Fig. 1). The exposed cells were prepared each day (starting on day 3) for ultrastructural radioautography by the flat substrate technique (*11*). At each time interval, 1000 cells through all layers of the plastic blocks (but not in adjacent sections) were scanned consecutively, first for the presence of silver grains over the nucleus and then for the presence in these labeled cells of filaments with forming Z bands or specific granules (secretory-like granules characteristic of atrial cardiocytes) (*12*). Only cells with these features (Fig. 2a) were considered to have synthesized DNA. The maximal incorporation of [^{3}H]thymidine occurred on day 5; at this time 63 percent of cardiocytes had labeled nuclei. This was followed by a sharp decline, with subsequent small peaks on days 9 and 11. Cells undergoing mitosis (Fig. 2b)—most of which were labeled—were always present but never exceeded 0.5 percent of the cardiocytes examined. A few labeled binucleate cells were encountered from day 5 on.

The entire spectrum of cell development, from very immature to nearly mature cardiocytes, was encountered in the cultures. Maturation of the cells was asynchronous; by day 7, cells with subsarcolemmal filaments accompanied by forming Z bands and an abundant rough endoplasmic reticulum were found side by side with more organized cells con-

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