the toxin in lipid bilayer membranes may not represent the primary mechanism of its biological action. Rather, they are a manifestation that the toxin molecules can span the membrane, and hence may be capable of interacting directly with cytoplasmic filamentous systems. Such interactions in addition to producing the morphological changes in tissue cultured neurons could also affect both transmitter release and synaptic vesicle recycling in presynaptic terminals. Conceivably, the toxin has two modes of action—the permeability changes induced by the channels cause transmitter release, whereas toxin interactions with cytoplasmic filamentous systems both inhibit recovery of synaptic vesicles from the axolemma and produce morphological changes in tissue cultured neurons.

Our results with lipid bilayers might lead to the prediction of nonspecific toxin effects on biological membranes (*13*). Yet studies on tissue cultured cells demonstrate that, while toxin produces striking morphological changes in several types of neurons, it does not affect nonneuronal cells—including glial cells, fibroblasts, macrophages, and muscle cells (*7*). Therefore, specificity of toxin action must reside either in differential toxin binding by specific cell types or in differential responses of cells to uniformly bound toxin. A still unanswered question is whether the toxin binds to specific proteins in the nerve membrane or only to lipids.

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13. The concentrations of toxin used on the bilayers are comparable with those used in the biological experiments.
14. This work was supported by NSF grant BMS 74-01139 to A.F., NIH grant NS 10883 to W. P. Hurlbut, and an NSF predoctoral fellowship to L.L.R.

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Modulation of C3b Hemolytic Activity by a Plasma Protein Distinct from C3b Inactivator

Abstract. *A human plasma protein binds to cell-bound C3b, the major cleavage product of the third component of complement. Consequent upon this binding, C3b no longer functions in either the classical or alternative pathways. This C3b inhibitory activity is a property of a protein previously designated β1H on the basis of its electrophoretic mobility.*

During complement activation, C3 (*1*) is enzymatically cleaved by either the classical or alternative pathway convertases into C3a and C3b. Biological activities of C3b include the enhancement of immune adherence and phagocytosis, continuation of the cytolytic sequence by reaction with C5 to C9, and generation of additional alternative pathway convertase by complexing with factor B and properdin. The C3b inactivator, an enzyme present in plasma, blocks these activities of C3b by cleaving it into two fragments, C3c and C3d (*2*). We have described a plasma protein, the C3b inactivator accelerator (A·C3bINA), that increases the rate at which the C3b inactivator inactivates C3b (*3*). We report here that, in addition to potentiating the activity of C3b inactivator, A·C3bINA directly binds to C3b and blocks its activities in both classical and alternative pathways.

A·C3bINA was purified (*4*) from the euglobulin fraction (*p*H 6.5) of human serum by NaCl gradient chromatography on QAE Sephadex A-50 in 0.01*M* tris-HCl containing 0.07*M* NaCl and 0.002*M* EDTA. The C3b inactivator was passed through the column with the starting buffer, and A·C3bINA was eluted, with the gradient at a conductivity of 11.0 reciprocal ohms (0°C). During subsequent chromatography on Bio-Rex 70 in 0.02*M* sodium phosphate, *p*H 7.0, A·C3bINA eluted with the NaCl gradient at a conductivity of 11.0 reciprocal ohms. In the final gel filtration step through Sephadex G-200 in 0.05*M* barbital, 0.14*M* NaCl, *p*H 7.5, A·C3bINA appeared at a volume corresponding to a molecular weight of approximately 300,000. From 1155 ml of plasma, containing 62 g of protein, 2.0 mg of A·C3bINA was obtained (*4*). On 4 percent polyacrylamide gel electrophoresis in 0.1 percent sodium dodecyl sulfate (SDS), a single dense band and two fainter bands were observed. The dense band contained more than 93 percent of the protein, as determined by staining with Coomassie blue and densitometry; its appearance was not altered by prior reduction of the sample with 0.2*M* β-mercaptoethanol in 8.0*M* urea. On standard polyacrylamide disc gel electrophoresis at *p*H 9.2 in the absence of SDS, A·C3bINA failed to enter the gel; a diffuse zone was observed in the upper 1 cm of gel. Immunoelectrophoresis of A·C3bINA against antiserum to normal human serum yielded a single arc of β mobility. Immunization of rabbits with 100 μg of purified A·C3bINA in Freund's complete adjuvant produced an antiserum that formed a dense precipitin arc identical to that observed with antiserum to normal human serum. A second faint arc in the alpha region was detected when this antiserum was used in immunoelectrophoresis against normal human serum: this arc was removed by treating the antiserum with a small amount of insolubilized (*p*H 6.5) pseudoglobulin (*5*). As shown below, the absorbed antibody neutralized the activity of A·C3bINA. In double diffusion, the monospecific antiserum produced reactions of nonidentity with antiserums to C1q, C4, C3, C5, C6, C7, C8, properdin, factor B, $C\bar{1}$ inhibitor, C3b inactivator, or any of 13 other antiserums to plasma proteins. A reaction of identity was observed with antiserum to β1H globulin (*6*), a protein previously named on the basis of

Table 1. Consumption of A·C3bINA by incubation with cellular intermediates. A·C3bINA (16.0 μg/ml of 0.01*M* EDTA-DGVB) was incubated with an equal volume of solution containing EA (2×10^8 per milliliter). EAC4 (1000 effective molecules of C4 per cell), EAC43 (1000 effective molecules of C3 per cell), or buffer for 1 hour at 37°C. After the cells had been removed by centrifugation, the residual A·C3bINA activity in the supernatants was assayed by the inhibition of formation of EAC43bPB from EAC43 bearing limited C3b sites (Fig. 1). The number of units is the reciprocal of the dilution required to inhibit 37 percent of the observed lysis (*15*).

Intermediate	Residual A·C3bINA activity (unit/ml)	Depletion (%)
EA	34	15
EAC4	22	45
EAC43	16	60
None	40	0

its immunoelectrophoretic mobility (*7*).

The effect of A·C3bINA on the hemolytic activity of the cellular intermediates EAC43, EAC1423, and EAC4 is shown in Fig. 1. Prior incubation with purified A·C3bINA produced a dose-dependent inhibition of the capacity of EAC43 prepared with limited C3b to interact with factor B and properdin to form the stable alternative pathway convertase, $\overline{C3bPB}$. The A·C3bINA also inhibited the ability of EAC1423 bearing limited C3b to lyse upon subsequent exposure to C5 to C9. At high concentrations of A·C3bINA, formation of the classical pathway convertase, $\overline{C42}$, from EAC4 bearing limited amounts of C4b was also impaired. In contrast to these effects on intermediates bearing C3b and C4b, prior incubation with A·C3bINA did not influence the hemolytic activity of EA prepared with limited antibody or EAC1 prepared with limited $\overline{C1}$.

The inhibitory effects of A·C3bINA on C3b and to a lesser extent on C4b are strikingly similar to those of the C3b inactivator, which also acts on both C3b and C4b (*8*). The two proteins were readily distinguished, however, by their behavior on ion-exchange or gel filtration chromatography, by the complete nonidentity of their precipitin reactions in agarose gel with their corresponding antibodies, and also by the ability of these antibodies to neutralize only the activities of their respective antigens. Excess amounts of purified $F(ab')_2$ fragments (*9*) of antiserum to A·C3bINA and antiserum to C3b inactivator were incubated at 37°C for 30 minutes with purified A·C3bINA or C3b inactivator. Each of the four reaction mixtures was then tested for residual inhibitory effect on C3b, with the use of EAC43 cells bearing limited C3b under the conditions for this intermediate given in Fig. 1. The antiserum to C3b inactivator removed 93 percent of the C3b inactivator activity, but only 5 percent of A·C3bINA activity, whereas antiserum to A·C3bINA removed 90 percent of the A·C3bINA activity but only 8 percent of the C3b inactivator. Thus the inactivation of cell-bound C3b mediated by A·C3bINA is not due to contaminating C3b inactivator, but is intrinsic to A·C3bINA itself.

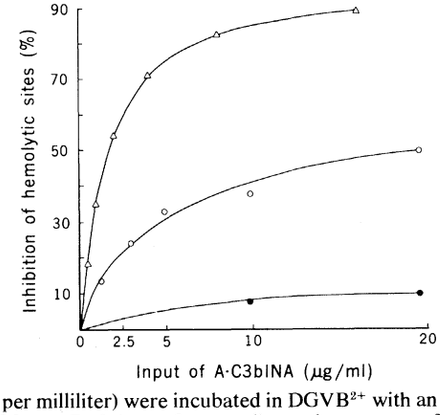

Fig. 1. Effect of A·C3bINA on the hemolytic activity of EAC43 (△), EAC1423 (○), and EAC4 (●). Cellular intermediates at a concentration of 1×10^8 per milliliter were exposed to equal volumes of A·C3bINA diluted in barbital-buffered saline with dextrose and gelatin (DGVB) containing 0.01*M* EDTA, *p*H 6.0, ionic strength, 0.037 (*13*), for 60 minutes at 37°C, washed once in barbital-buffered saline with dextrose, gelatin, Ca^{2+}, and Mg^{2+}, *p*H 7.5, ionic strength, 0.075 ($DGVB^{2+}$) (*2*), and then resuspended in $DGVB^{2+}$ for the developing reactions. Cellular intermediates were prepared with guinea pig C1, human C4, C2 (oxidized for EAC1423), and C3 according to published methods (*2*). *For EAC43*, 0.9 effective molecule of C3 per cell was used as assayed in the following system: cells (1×10^8 per milliliter) were incubated in $DGVB^{2+}$ with an equal volume of $DGVB^{2+}$ containing 0.7 μg of factor B, 0.1 μg of properdin, and an excess of factor $\overline{D}$, to allow formation of $\overline{C3bPB}$. Hemolysis of cells bearing this enzyme was effected by the addition of an excess of C3 to C9 in the form of 0.3 ml of rat serum diluted 1/15 in 0.04*M* EDTA-GVB (*2*). Average numbers of effective molecules of C3b per cell were calculated from measurements of hemoglobin released (*2*). *For EAC1423*, 0.67 effective molecule of C3 per cell was used as assayed by development at 37 °C for 60 minutes with 0.2 ml of $DGVB^{2+}$ containing 50 units of C5 per milliliter and excess C6 to C9 in the form of 1/200 dilution of KSCN-treated guinea pig serum (*14*). *For EAC4*, 0.82 effective molecule of C4 per cell was used, assayed as follows: EAC4 (1×10^8 per milliliter) were incubated in $DGVB^{2+}$ at 30°C for 15 minutes with an equal volume of $DGVB^{2+}$ containing 400 units of $C\bar{1}$ per milliliter, then at 30°C for 10 minutes with an additional volume of $DGVB^{2+}$ containing 50 units of C2 per milliliter; then the presence of $\overline{C42}$ on the cells was determined as for $\overline{C3bPB}$ above.

Table 2. Agglutination by antiserum to A·C3bINA of cells exposed to varying concentrations of A·C3bINA. EAC43 (1000 effective molecules of C3 per cell), EAC4 (1000 effective molecules of C4 per cell), or EA (1×10^8 per milliliter) was incubated in 0.01*M* EDTA-DGVB with equal volumes of varying concentrations of A·C3bINA for 60 minutes at 37°C. The cells were washed once in barbital-buffered saline containing 0.1 percent bovine serum albumin (SAVB), and resuspended in SAVB to 1×10^8 per milliliter. Antibody to A·C3bINA was serially diluted in 0.025 ml of SAVB in microtiter plates, one drop (0.025 ml) of cell suspension was added to each well, and sedimentation was allowed to occur at room temperature over a period of 4 hours.

Input of A·C3bINA (μg/ml)	Agglutination titer		
	EAC43	EAC4	EA
16	1/3200	1/40	<1/10
8	1/1600	1/20	
4	1/800	1/20	
0	<1/100	<1/10	<1/10

The observation that the inhibitory effects of A·C3bINA on EAC43 bearing limited C3b persisted after the cells had been washed (Fig. 1) suggested that A·C3bINA might bind directly to the cellular intermediate. Depletion of A·C3bINA from the fluid phase demonstrated in the experiment shown in Table 1 was consistent with this hypothesis: incubation of A·C3bINA with EAC4 or EAC43 led to 45 and 60 percent loss of activity from the supernatants, respectively. A second experiment, in which antibody to A·C3bINA was found to agglutinate EAC43 that had been incubated with A·C3bINA (Table 2) demonstrated that A·C3bINA was physically bound to the treated cells. Consistent with the weak interactions with C4b (Fig. 1 and Table 1), low titer agglutination of A·C3bINA-treated EAC4 was also observed.

The ability of A·C3bINA to interact with C4b resembles the action of a 10*S* globulin which must necessarily be present simultaneously with C3b inactivator in order for C4b to be cleaved to C4c and C4d (*10*). It has been shown (*3*) that A·C3bINA also had to be simultaneously present with C3b inactivator to potentiate the activity of this enzyme on C3b. This effect is observed at A·C3bINA concentrations that are one-tenth of those required for the direct effects reported above. The concentration of A·C3bINA in plasma, as estimated from radial immunodiffusion with monospecific antibody, is of the order of 150 to 300 μg/ml, so that the direct effects would operate under physiologic conditions.

A·C3bINA also resembles a "C5 site

generation inhibitor" found in guinea pig serum by Okada *et al.* (*11*). This inhibitor, which is believed to form a complex with cell-bound C3b and inhibit the generation of the C5 binding site, elutes from Sephadex G-200 in a position similar to A·C3bINA. It does not block the immune adherence activity of C3b; we have some data to indicate that A·C3bINA also does not block immune adherence.

A·C3bINA is the third plasma protein described which forms complexes with C3b, the others being properdin and factor B (*12*). The binding of the last two leads to activation or stabilization of the alternative pathway convertase. Binding of A·C3bINA prevents the participation of C3b in both the alternative and classical pathways.

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Sialic Acid: Effect of Removal on Calcium Exchangeability of Cultured Heart Cells

Abstract. *Specific removal of sialic acid from cultured heart cells with purified neuraminidase increases cellular calcium exchangeability. Potassium exchange is unaffected or slightly decreased. Sialic acid removal also permits lanthanum, normally restricted to the cellular surface, to enter the cells and displace more than 80 percent of cellular calcium. The results indicate a specific role of cellular surface components in the control of calcium exchangeability in the heart.*

The surfaces of most animal cells are negatively charged. A major source of this negativity in cells from diverse tissues is the ionized carboxyl groups of sialic acid (*1*). The sialic acids occupy peripheral, terminal positions in the oligosaccharide portions of the glycoproteins and glycolipids that make up a large component of the coat material of the cell surface (*2*). Sialic acid accounts for a considerable component of cation binding on the cell surface with a particular affinity for calcium as demonstrated for red blood cell membrane (*3*).

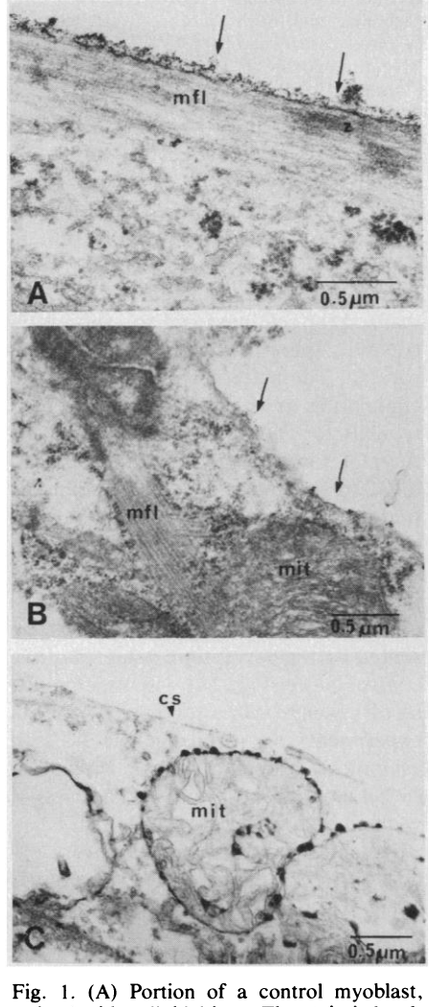

Fig. 1. (A) Portion of a control myoblast, stained with colloidal iron. The stain is localized to the cell surface (arrows); *mfl*, myofilaments; *Z*, Z-line material (× 30,000). (B) Portion of myoblast treated with neuraminidase, then stained with colloidal iron. There is an almost complete lack of staining of cell surface (arrows); *mfl*, myofilaments; *mit*, mitochondria (× 26,000). (C) Portion of a myoblast treated with neuraminidase and then stained with La. Note the intracellular accumulation of La around the mitochondria (*mit*) outer membrane; *cs*, cell surface (× 24,000).

The ability of the trivalent cation lanthanum (La^{3+}) to bind at the surface of cultured heart cells, displace significant quantities of calcium (Ca^{2+}), and inhibit Ca^{2+} exchange (*4*) suggested that negatively charged molecules, specifically sialic acid, might be of importance in the regulation of Ca^{2+} permeability in the cells.

Colloidal iron applied at *p*H 1.7 is a specific stain for sialic acid (*5*); it was used to show the distribution of sialic acid at the cell surface (Fig. 1A) by electron microscopic techniques (*4*). The specificity of the technique was demonstrated by the almost complete elimination of the stain density (Fig. 1B) when the cells were exposed to a purified neuraminidase preparation (*6*). At concentrations applied to the cells the enzyme was assessed for protease activity with ^{125}I-labeled albumin as substrate. No proteolytic activity was detectable; phospholipase activity of the enzyme preparation was measured by incubating the neuraminidase for 90 minutes with [^{14}C]phosphatidylcholine as substrate. The extent of hydrolysis was compared to that obtained with phospholipase C, and the neuraminidase was found to contain the equivalent of 0.0025 unit of phospholipase activity per milliliter. Cultured heart cells (from newborn rats) were then incubated with 0.0025 unit of phospholipase per milliliter under conditions identical to those used with neuraminidase incubation. No effect on beating or on ^{45}Ca exchangeability (see below) of the cells was produced.

The ^{45}Ca exchange of the cultured cells was continuously monitored with the scintillation-disk flow cell technique (*4*), which depends on the growth of a cellular monolayer on one surface of each of two disks composed of polystyrene combined with scintillation material (*7*). The cells were cultured by a standard trypsin digestion technique (*8*). The proportion of myoblasts was increased by in-