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ISOLATION OF AN iC3b FORMING ENZYME FROM PERITONEAL POLYMORPHONUCLEAR LEUKOCYTES OF GUINEA PIGS

Tsukasa Seya and Shigeharu Nagasawa

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

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SUMMARY: We have investigated fluid phase cleavage of C3b by peritoneal polymorphonuclear leukocytes of guinea pigs and found that polymorphonuclear leukocytes expressed an iC3b forming enzyme as well as C3b receptor with maturation in peritoneal cavity. The iC3b forming enzyme was found to be distinct from C3bINA, a physiological iC3b forming enzyme in plasma, since the activity was inhibited by monoiodoacetic acid and did not require a cofactor plasma protein, βlH, for the cleavage of C3b into iC3b. The iC3b forming enzyme is gradually released upon incubation of PMN at 37°C. The molecular weight of the iC3b forming enzyme was estimated to be 48,000 from gel filtration on Sephadex G-200.

The activation fragment of the third component of complement, C3b, has been shown to play an important role in the classical and alternative pathways of complement system; it forms a C3 converting enzyme with factor B and thus amplifies activation of the alternative pathway of complement system (1); it allows the C3 converting enzymes of both pathways to cleave C5 (2); it interacts with a C3b-receptor, CR1, on blood cells and triggers a sequence of reactions which lead to respiratory burst, phagocytosis, and immune adherance(3).

These functions of C3b are partly regulated by C3b INA, which together with a cofactor plasma protein, βlH, cleaves C3b into a nicked C3b derivative, iC3b (4). Although iC3b is devoid of the activity to assemble C3 and C5 convertases, it still retains an ability to interact with a C3 receptor, CR3 (5). There is

Abbreviations used: PMN, polymorphonuclear leukocyte; DACM, N-(dimethylamino-4-methylcoumarinyl)-maleimide; DFP, diisopropyl-fluorophosphate; SDS, sodium dodecyl sulfate

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increasing evidence suggesting that the interaction between iC3b and CR3 on phagocytes is required for the phagocytosis of C3b-coated particles (6). Thus, it was of interest whether phagocytes have a specific protease and convert C3b into iC3b thereby enhancing cellular functions of phagocytes.

The present paper documents that a novel thiol protease , which converts C3b into iC3b just as C3bINA, increases on the surface of PMN upon maturation of PMN in the peritoneal cavity of guinea pigs. The iC3b forming enzyme differs from C3bINA in the point that it is inhibited by monoiodoacetic acid and does not require cofactor proteins for the cleavage of C3b into iC3b.

MATERIALS AND METHODS

Following materials were obtained as indicated: a fluorescent thiol reagent, N-(dimethylamino-4-methylcoumarinyl)-maleimide (DACM) and casein sodium from Wako Pure Chemicals, Japan; diisopropylfluorophosphate (DFP) from Kishida Chemicals, Japan; Sephadex G-200 from Pharmacia, Sweden; Ampholine from LKB, Sweden.

Complement components, C3b (7) and C3bINA (8) were prepared from human plasma, according to the published methods. βlH was obtained as the by-product of C3 purification (7). The C3b and C3bINA preparations were further treated with anti- βlH-antibody Sepharose to remove βlH in the preparations. The complement components were dialyzed against Krebs-Ringer-phosphate buffer , pH 7.2. Protein concentrations of complement components were estimated from their absorbance, based on a value of 10.0 for A 280 of the 1% solution.

Preparation of PMN. Guinea pigs were injected intraperitoneally with 15 ml of 3% casein sodium. After 14-42 hr, the peritoneal cells were harvested and isolated according to the method reported by Washida et al. (9). More than 85% of the cells were found to be PMN by May-Gruenwalds-Giemsa's staining method.

Fluorometry for determinations of the iC3b forming activity and C3b receptor. The thiol residue of C3b was labeled with DACM (10) and the iC3b forming activity was determined with the fluorescent C3b as follows; 200 µl (18µg) of DACM-C3b and 100 µl(10^7 cells) of PMN were incubated for 60 min at 37°C and centrifuged for 4 min at 200xg. The supernatant fraction thus obtained was reduced with 10µl of 10% SDS-30% 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis (11). The gel was scanned in a Hitachi fluorospectrophotometer under excitation at 385 nm and emission at 475 nm. Since cleavage of DACM-C3b into DACM-iC3b is accompanied by the cleavage of the fluorescent-labeled α chain (Mr 110000) into a fluorescent-labeled α-1 fragment (Mr 70000) and non-fluorescent α -2 fragment (Mr 40000)(10), the iC3b forming activity can be estimated from the percent cleavage of the α chain ;

$F\alpha 1/(F\alpha + F\alpha 1)$, where Fα and Fα1 were fluorescence intensities of α and α 1 fragment, respectively.

CR1 was determined by its cofactor activity upon C3bINA-mediated cleavage of C3b into iC3b as reported by Sim and Sim (12); DACM-C3b (18μg), PMN (10^7 cells), and C3bINA (1μg) were incubated for 60 min at 37°C and the percent cleavage of C3b was determined as described above. The increased cleavage of iC3b upon addition of C3bINA to PMN was taken as the apparent CR1 site.

Isoelectric focusing was performed with Ampholine of pH range of 3-10 in 6 % polyacrylamide gel(13). The gel was sectioned into 3 mm segments, extracted with water for pH measurement or with 0.15M phosphate buffer, pH 7.5 for determination of the iC3b forming activity.

RESULTS

Detection of a PMN-associated iC3b forming activity

Peritoneal PMN were harvested periodically after the peritoneal injection of casein into guinea pigs and was determined for the activity which cleaved C3b into iC3b. As shown in Fig. 1, the cleavage of C3b into iC3b was found to occur upon incubation of C3b with peritoneal PMN. Interestingly enough, the PMN harvested 24-42 hr after casein injection showed almost 4-fold increased iC3b forming activity, compared with the PMN harvested 14 hr after casein injection. The activity was not enhanced by the addition of β1H, a cofactor protein of C3bINA. In addition, when the apparent CR1 site was determined by measuring its cofactor activity on C3bINA-mediated C3b cleavage(12), this CR1 site was

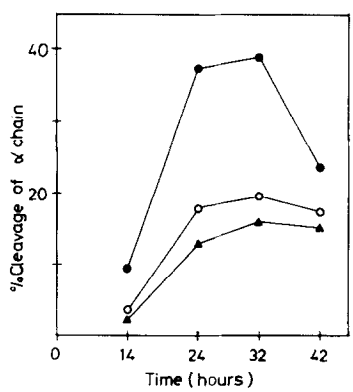


Fig.1. Time courses of appearance of an iC3b forming activity and C3b receptor on PMN in the peritoneal cavity of guinea pigs. PMN was harvested from the peritoneal cavity at indicated times after the peritoneal injection of casein. C3b cleavage was performed with PMN alone (○), PMN and 1μg C3bINA (●), and PMN and 4μg β1H (▲).

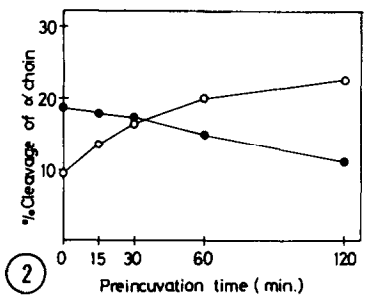
also found to increase with prolongation of the period before harvest of PMN from the peritoneal cavity. These results suggest that the iC3b forming enzyme as well as CR1 are increasing or fully expressing during maturation of PMN in the peritoneal cavity.

Release of the iC3b forming enzyme from PMN

When PMN was in vitro incubated at 37°C, the iC3b forming activity was gradually released into the medium , and the PMN-associated activity was inversely decreased. (Fig. 2). Addition of β1H or C3bINA to the medium did not enhance the iC3b forming activity, suggesting that neither CR1 nor C3bINA was released from PMN.

Characterization of the iC3b forming enzyme

PMN ($3x10^8$) harvested 24 hr after casein injection were incubated for 2 hr at 37°C and the supernatant fraction was applied to a column of Sephadex G-200. As shown in Fig. 3, the



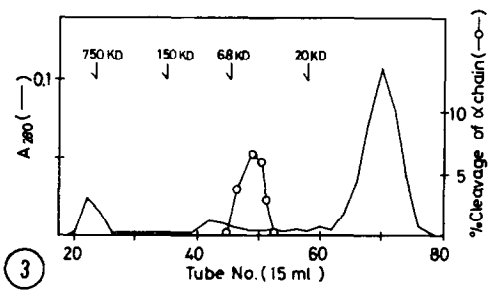


Fig.2. Release of the iC3b forming enzyme from PMN. The PMN (10^8 cells/ml) harvested 24 hr after casein injection were incubated at 37°C. At the indicated times, 100μl of the cell suspension was removed and centrifuged for 5 min at 200xg. The iC3b forming activity was determined with the supernatant (O) and the cell pellets (●).

Fig. 3. Gel filtration of the iC3b forming enzyme released from PMN on Sephadex G-200. Ten ml of the PMN suspension (10^8 cells/ml) harvested from four guinea pigs 24 hr after casein injection were incubated for 2 hr at 37°C and centrifuged for 5 min at 200xg. The supernatant fraction was applied to a column (4.5 x 75 cm) of Sephadex G-200 equilibrated with phosphate buffered saline, pH 7.4. The iC3b forming activity was determined with each 100 μl of eluates. The column was calibrated for molecular weight with a mixture of guinea pig serum and soybean trypsin inhibitor.

iC3b forming activity was eluted as a peak of Mr 48,000. The activity was inhibited by 10 mM monoiodoacetic acid but not by 2 mM DFP and 10 mM EDTA, suggesting the enzyme to be a thiol protease. The isoelectric point of the enzyme was determined to be 7.5 and the pH optimum of the activity was found to be 6.0.

DISCUSSION

The present study demonstrates that peritoneal PMN of guinea pigs contain a novel thiol protease which cleaves C3b into iC3b and that the iC3b forming enzyme appears to increase , together with CR1, upon maturation of PMN in the peritoneal cavity. The peritoneal PMN collected 14 hr after casein injection have been generally used in inflammatory and immunological researches (9,14). Interestingly enough, the PMN thus collected after 14 hr showed only low level of the activity and it takes over 24 hr until PMN fully expressed the iC3b forming activity. The increased expression of CR1 was reported to occur when human PMN was subjected to mechanical forces during cell separation or treatment with various chemoattractants such f-Met-Leu-Phe, C5a, and casein (15). However, the low level of iC3b forming activity of the PMN collected after 14 hr was not enhanced after incubation for 2 hr with the peritoneal fluid collected after 32 hr. The reason why over 24 hr is required for full expression of the activity on PMN remains to be solved. Also, whether the increase of the iC3b forming activity means the activation of a precursor form or the biosynthesis of the protease remains unclear. The iC3b forming enzyme is inhibited not by DFP and EDTA but by monoiodoacetic acid. This property excludes a possibility that the iC3b forming activity is due to well-known lysosomal proteases, such as elastase, cathepsin G, and collagenase (16-18). Three types of lysosomal thiol proteases, such as cathepsins B, H, and L, nave been isolated. The molecular weights

of these thiol proteases are reported to be in the range of 21,000-28,000 (19) and are lower than that of the iC3b forming enzyme, 48,000. The thiol protease nature and the molecular weight of the iC3b forming enzyme also exclude a possibility that the enzyme is C3bINA itself of guinea pig plasma. In addition, C3bINA requires a cofactor protein for its function, but the iC3b forming enzyme does not require it.

Since iC3b is far more susceptible to proteases than C3b, digestion of C3b with various proteases, such as plasmin, elastase, and trypsin, usually proceeds to cleavage of iC3b into C3c and C3d and therefore iC3b is not detected as an intermediate product (7,20,21). Thus, evidence that the iC3b forming enzyme does not cleave iC3b into C3c and C3d suggests that the iC3b forming enzyme is of restricted substrate specificity, such as C3bINA.

Wright and Silverstein reported that iC3b receptor was 3-10 times more efficient in promoting attachment or ingestion of C3b-coated particles(22). Thus, the iC3b forming enzyme on PMN may cleave C3b-coated particles to yield iC3b-coated particles and enable the interaction between CR3 and iC3b-coated particles thereby accelerating the processing of the C3b-coated particles by phagocytes.

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