Inhibition of Collagen-Induced Platelet Aggregation by Antibodies to Distinct Types of Collagens

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Aggregation of platelets by fibrils formed from collagens type I, II and III could be inhibited by coating the fibrils with anti-collagen antibodies or Fab fragments. Similar results were obtained in a clot-retraction assay. Inhibition was achieved with stoichiometric amounts of antibodies and was specific for each type of collagen. Aggregation caused by a mixture of type-I and -III collagens could only be inhibited by a mixture of antibodies against both collagens. The data show that each interstitial collagen is capable of interacting with platelets and do not support the concept of an outstanding activity of type-III collagen.

Aggregation of platelets by collagen is considered as a useful system *in vitro* for studying the adherence of platelets to subendothelial structures in injured blood vessels (Michaeli & Orloff, 1976). Previous studies have established that soluble collagens are poorly active in aggregation, but become very efficient when precipitated in the form of fibrils (Muggli & Baumgartner, 1973; Gordon & Dingle, 1974; Jaffe & Deykin, 1974; Brass & Bensusan, 1974; Balleisen *et al.*, 1975a; Barnes *et al.*, 1976). Collagen exists in the body in several polymorphic variants, and three genetically distinct types, I, II and III, are well characterized (Fietzek & Kühn, 1976; Miller, 1976). These types of interstitial collagens were all able to aggregate platelets, but type-III collagen was on a weight basis more active than the other two collagens (Balleisen *et al.*, 1975a). This finding correlated with immunohistological data suggesting a high content of type-III collagen in the subendothelial region of the vessel wall (Gay *et al.*, 1975). The strong interaction between platelets and type-III collagen was confirmed by Hugues *et al.* (1976), and these authors concluded that the lower activity found for type-I collagen was not an inherent property of this protein, but could be due to contamination by type-III collagen.

Since aggregation of platelets depends on a particular quaternary structure of collagen, it is difficult to explore the mechanism at the molecular level. Monovalent antibody fragments to fibrinogen were successfully used to study platelet aggregation caused by thrombin (Tollefsen & Majerus, 1975).

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We have previously described specific antibodies to type-I, -II and -III collagens (Nowack *et al.*, 1976). Here, we used these antibodies to characterize platelet aggregation by various types of collagens, using a specific inhibition assay.

Materials and Methods

Preparation of collagen fibrils

Type-I and -III collagens were extracted with 1 M-NaCl from foetal bovine skin and purified by salt precipitation and chromatography on DEAE-cellulose (Timpl *et al.*, 1975). As judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, contamination of type-I collagen by type-III collagen was negligible, and vice versa. Type-II collagen was solubilized from bovine articular cartilage by limited digestion with pepsin and purified as described by Miller (1972). Solutions of these collagens in 0.1 M-acetic acid (0.5 mg/ml) were dialysed at 4–8°C against 0.02 M-sodium phosphate buffer, pH 7.4. During dialysis collagen formed an opaque suspension of fibrils, which, as shown previously (Balleisen *et al.*, 1976), was very efficient in aggregating platelets.

Purification of antibodies and Fab fragments

Purified antibodies were prepared from rabbit antisera against type-I, -II and -III collagens and rendered specific for the antigen by immunoabsorption. As judged by agglutination and radioimmunoassays, the antibodies showed a strong reaction with the collagen used for immunization and did not cross-react with the other two types of collagen (Nowack *et al.*, 1976). Antibodies to bovine serum albumin and bovine fibrinogen were prepared by a similar immunoabsorption procedure. Immunoglobulin G

was purified from a rabbit antiserum to type-I collagen on DEAE-cellulose and then digested with mercuripapain (Worthington, Freehold, NJ, U.S.A.) by an established procedure (Edelman & Marchalonis, 1967). Fab fragments were isolated from the digest by immunoabsorption on type-I collagen (Beil *et al.*, 1973). The fragments were further purified on Bio-Gel P-100 and were found by gel diffusion not to contain Fc antigen. All antibody solutions were finally dialysed against 0.02M-phosphate buffer, pH7.4.

Immuno-electron-microscopy of antibody-coated collagen fibrils

A suspension of collagen fibrils (0.2mg/ml) in 0.02M-phosphate buffer, pH7.4, was incubated with an equal volume of purified antibody (0.2mg/ml) for 30min at 20°C and non-bound antibody was subsequently removed by centrifugation (1000g for 10min). The fibrils were washed three times with 0.02M-phosphate buffer, pH7.4, resuspended in the buffer and incubated with peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (Miles Laboratories, Kankakee, IL, U.S.A.). Peroxidase was then detected by reaction with 3,3'-diaminobenzidine as oxidizable substrate (Graham & Karnovsky, 1966). After a further staining of the fibrils with phosphotungstic acid they were examined in the electron microscope.

Platelet-aggregation assay

For the preparation of platelet-rich plasma, blood was collected from healthy volunteers and prevented from clotting by adding 0.1 vol. of 3.8% (w/v) sodium

citrate. After centrifugation at 90g for 10min the supernatant fluid was collected and contained 200000–400000 platelets/ μ l. Platelet aggregation was determined in an aggregometer as described by Born & Gross (1963). Increase in light transmission was monitored by a linear recorder (Vitatron UR 400). In the reaction 0.45ml of platelet-rich plasma was mixed with 0.05ml of a suspension of collagen fibrils and incubated at 37°C. The minimal aggregation dose of each batch of collagen fibrils was determined as described previously (Balleisen *et al.*, 1975a). In the inhibition assay 1.5–2 times the minimal aggregating dose of fibrils was incubated for 5min at room temperature with various amounts of purified antibodies or Fab fragments before addition to the platelets. No difference in the results was observed either when the whole mixture of fibrils and antibodies was added to the reaction or when non-bound antibody was removed from the fibrils by centrifugation.

Fibrin-clot retraction assay

Platelet-rich plasma (0.7ml) was mixed with 0.1ml of collagen fibrils (200 μ g/ml) and 0.2ml of a solution of Arvin in 0.15M-NaCl (12 μ g/ml). Arvin (Knoll A.G., Ludwigshafen, Germany) is a snake venom from Malayan pit viper (*Agkistrodon rhodostoma*) and resembles in its activity the snake enzyme reptilase, i.e. it causes fibrin-clot formation by release of fibrinopeptide A (Hessel & Blombäck, 1971). Unlike thrombin, it does not aggregate platelets by itself (Sharp *et al.*, 1968). The mixture was incubated at 37°C and the size of the clot was measured at various intervals between 15 and 200min of incubation.

Fig. 1. Inhibition by antibodies of platelet aggregation induced with 0.5 μ g of type-III collagen (a) or with 2 μ g of type-I collagen (b)

Antibodies in the amounts indicated were against type-I collagen (anti-I), type-II collagen (anti-II), type-III collagen (anti-III) or bovine serum albumin (anti-BSA); controls without antibody were denoted by 0 μ g.

EXPLANATION OF PLATE 1

Observation of antibody coating of collagen fibrils by the indirect antibody-oxidase technique

(a), Type-I collagen fibrils stained with antibodies against type-I collagen; (b), same as (a), but stained with antibodies against type-III collagen; (c), type-III collagen fibrils stained with antibodies against type-III collagen; (d), same as (c), but stained with antibodies against fibrinogen. The second antibody was a peroxidase conjugate of goat anti-(rabbit immunoglobulin G).

Fig. 2. (a) Inhibition by antibodies of platelet aggregation induced with a mixture of type-I and type-III collagens; (b) comparison of the inhibiting activities of antibodies and Fab fragments
The abbreviations are the same as in Fig. 1.

Results

Reaction of antibodies with collagen fibrils

Antibodies to various types of collagens show a strong and specific reaction with soluble collagens in passive haemagglutination and radioimmunoassays (Nowack *et al.*, 1976). We have now examined the reaction of the same antibodies with collagen fibrils by electron microscopy by using the indirect antibody-peroxidase technique. Plates 1(a) and 1(c) show that type-I collagen and type-III collagen fibrils could be coated by antibodies to type-I or type-III collagen respectively. The reaction was specific, since antibodies to type-III collagen did not stain type-I collagen fibrils (Plate 1b), nor did anti-fibrinogen antibodies react with any type of collagen fibrils (Plate 1d). The benzidine stain on the coated type-I collagen fibrils appeared to have some regular spacing, whereas a more compact stain was observed on type-II and -III collagen fibrils. This may reflect density of antigenic determinants on the fibrils. Antisera to type-I collagen possess mainly antibodies to a single determinant at the C-terminal end of the molecule (Becker *et al.*, 1972), whereas multiple determinants are recognized in the antibody response to type-II and -III collagens (Hahn *et al.*, 1975; H. Nowack & R. Timpl, unpublished work).

Inhibition of platelet aggregation by anti-collagen antibodies

Type-III collagen fibrils were very efficient in aggregating platelets at a concentration as low as 0.5 µg/ml. Addition of increasing amounts of antibodies against type-III collagen produced an increasing inhibition of the reaction, and no aggregation was observed after incubation with 2 µg of antibody

Fig. 3. Inhibition by antibodies against type-II collagen of clot retraction caused by type-II collagen (20 µg)
The amounts of antibodies added are indicated in the Figure. Control denotes the change in the clot in the absence of collagen and antibodies.

(Fig. 1). Addition of antibodies against type-I collagen, fibrinogen or serum albumin had no effect on the reactions. Inhibition results with comparable specificity were obtained for the aggregation reaction with type-I collagen fibrils (Fig. 1) and with type-II collagen. Only antibodies against the particular collagen used in the tests showed complete inhibition, whereas unrelated antibodies were not inhibitory even when used in 2-3-fold higher amounts.

We also examined inhibition of aggregating properties of fibrils formed from a solution containing both type-I and type-III collagen. As shown in Fig. 2(a), antibodies to neither type-I collagen nor type-III collagen alone could substantially inhibit the reaction, but a mixture of both antibodies was very efficient. Finally, we have studied monovalent Fab fragments obtained from anti-collagen antibodies and found them to be as active as the intact antibodies (Fig. 2b).

Clot retraction by collagen and its inhibition by antibodies

Fibrin clots formed in the presence of platelets may retract on addition of various aggregating substances (Nyman, 1973). We found that fibrils prepared from various types of collagens also cause clot retraction, as illustrated for type-II collagen in Fig. 3. Here again, clot retraction could be increasingly inhibited by adding increasing amounts of antibodies to type-II collagen. No effect was observed when antibodies to unrelated antigens were added to this reaction.

Discussion

The data demonstrate that platelet aggregation caused by collagen fibrils could be specifically blocked by coating the fibrils with antibody or Fab fragments. This is in some ways a surprising observation, since soluble immune complexes or aggregated immunoglobulins are usually very potent in aggregating platelets (Lüscher *et al.*, 1973). The interaction is thought to be mediated by Fc receptors in the platelet membrane (Israels *et al.*, 1973) and can be inhibited to some extent by monomeric immunoglobulin. Presumably the antibodies are arranged on the surface of collagen fibrils in a special array, rendering them incapable of interaction with Fc receptors but still interfering in the interaction of collagenous structures with platelets by either direct blockade or steric hindrance. It remains to be determined whether further increasing the antibody coat on the fibrils is possible, and whether this could then result in an immune complex capable of aggregating platelets. From the experiments with Fab fragments and with mixtures of antibody-coated and non-coated fibrils (Fig. 2), it can be excluded that antibody-fibril complexes remove, via their Fc sites, secondary factors from the plasma which might be required for efficient collagen-platelet aggregation such as found in some studies with immune complexes (Lüscher *et al.*, 1973). Antibody inhibition of collagen-induced platelet aggregation is also basically different from inhibition of thrombin-induced aggregation by anti-fibrinogen Fab fragments. The fragments do not interfere with proteolytic conversion of surface-bound fibrinogen, but do with polymerization of fibrin (Tollefsen & Majerus, 1975).

As shown previously, type-I, -II and -III collagen fibrils may vary to some extent in their efficiency to aggregate platelets (Balleisen *et al.*, 1975a; Hugues *et al.*, 1976; Barnes *et al.*, 1976). It was speculated that type-I collagen is actually inactive, and various extents of type-III collagen contamination in the preparations are responsible for the interaction with platelets (Hugues *et al.*, 1976). The specificity of antibody inhibition shown here correlates well with previous immunochemical data (Nowack *et al.*, 1976), and clearly demonstrates that each type of interstitial collagen is able to aggregate platelets. Our data also agree with previous findings where highly purified $\alpha 1(I)$ chains from type-I collagen have been found to aggregate platelets after appropriate renaturation and fibril formation (Balleisen *et al.*, 1976; Fauvel *et al.*, 1978a). Some forms of collagens derived from basement membranes were found not to be active in aggregation (Trelstad & Carvalho, 1979), whereas other collagens such as type AB₂ are active (L. Balleisen & J. Rauterberg, unpublished work).

Antibody coating apparently does not distort the gross structure of the fibrils (Plate 1), indicating that antibodies do not prevent trapping of cells by tightening a loose meshwork of fibrils rather than by competing for collagenous structures at or close to specific platelet-binding sites. The identification of such binding sites was attempted by using collagen CNBr peptides in assays other than direct platelet aggregation, since such peptides cannot be examined in fibrillar form. Activity was mainly found in peptide $\alpha 1(III)$ -CB4 of type-III collagen (Fauvel *et al.*, 1978b; L. Balleisen, unpublished work), whereas results for type-I collagen are still controversial and show activity in the C-terminal peptide $\alpha 1(I)$ -CB6 (Balleisen *et al.*, 1975b; Fauvel *et al.*, 1978a) as well as in the glycopeptide $\alpha 1(I)$ -CB5 (Chiang & Kang, 1976). These data do not necessarily indicate that the same sites are involved in the aggregation reaction. The preparation of antibodies to single antigenic determinants of collagen is feasible (Timpl, 1976) and should allow localization by inhibition assays of such sites in fibrils, particularly when Fab fragments are used instead of the bulkier antibodies.

Native fibrils formed *in vitro* from either type-I or type-III collagen do not differ in cross-striation banding (Wiedemann *et al.*, 1975). It is therefore still impossible to decide by electron microscopy whether both collagens are assembled *in vitro* or *in vivo* in independent fibrillar structures. Immunofluorescence has indicated that type I and -III collagens in tissues are localized in different anatomical regions (Timpl *et al.*, 1977), but these studies have not yet been extended to the ultrastructural level. Antibody inhibition of platelet aggregation by fibrils prepared from a mixture of type-I and -III collagens (see Fig. 2) suggests that formation of mixed-type fibrils has not occurred to a substantial degree. Thus specific anti-

body reagents may be versatile tools with which to study various biological properties of interstitial collagens.

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