Inhibition of Platelet Aggregation by Monoclonal Antibody Reactive with β_2-Microglobulin Chain of HLA Complex

Abstract. *A mouse monoclonal antibody that reacts with β_2-microglobulin, the light chain of class I major histocompatibility antigens, inhibited the second wave of human platelet aggregation induced by adenosine diphosphate and epinephrine and blocked aggregation and platelet protein phosphorylation induced by sodium arachidonate. Thrombin-induced platelet aggregation was inhibited at threshold concentrations but not at higher concentrations. The antibody also inhibited aggregation and secretion in response to thromboxane A_2 or the stable endoperoxide analog, U46619. These results suggest that β_2-microglobulin in the histocompatibility complex is intimately associated with transmission of the endoperoxide-thromboxane signal at the platelet membrane.*

The second wave of platelet aggregation and platelet secretion are related events in which products of the cyclooxygenase pathway of arachidonate metabolism play an important role. Inhibition of platelet cyclooxygenase by acetylsalicylic acid blocks the formation of endoperoxides and thromboxane A_2 (TXA_2) and abolishes platelet secretion and the secondary wave of platelet aggregation (*1*). The addition of either endoperoxides or TXA_2 to platelets incubated with acetylsalicylic acid results in aggregation and secretion which includes the release of serotonin and adenine nucleotides (*1*). The formation and inhibition of endoperoxides and TXA_2 are subjects of interest because of the potential adverse effects of their formation in atheromatous vasculature (*2*). We have produced a monoclonal antibody, UMR-304, which inhibits the second wave of aggregation in platelet-rich plasma treated with sodium citrate to prevent coagulation (C-PRP) (*3*). This antibody has now been demonstrated to inhibit human platelet aggregation induced by both an endoperoxide analog and TXA_2.

The monoclonal antibody UMR-304 was produced by fusion between the nonsecreting murine myeloma line P3-NS1-AG4-1 and BALB/c mouse spleen cells sensitized to human peripheral blood lymphocytes. The first indication of the specificity of UMR-304 came from its failure to react with Daudi cells. Daudi is a human lymphoblastoid cell line which does not express β_2-microglobulin or HLA-A or HLA-B allotypic antigens on its surface (*4*). β_2-Microglobulin (β_2m) is the 11,818 dalton common chain of the class I (HLA) histocompatibility antigens. UMR-304 was found to react with β_2m in an indirect enzyme linked immunosorbant assay (ELISA) in which highly purified β_2m (*5*) was used as the antigen. UMR-304 was then affinity purified on β_2m–Sepharose 4B columns. Purified antibody reacted with β_2m in ELISA. It was positive in indirect immunofluorescence with peripheral blood mononuclear cells, platelets, cultured cell lines RPMI 1788, Raji, Molt-4, and CCRF-CEM, but not Daudi cells. UMR-304 (10 to 20 μg/ml) abolished second wave aggregation of human C-PRP induced by adenosine diphosphate (ADP) or epinephrine. Aggregation of human gel-filtered platelets induced by 0.1 to 0.2 unit of thrombin was not inhibited by 10 μg of UMR-304 per milliliter; however, at threshold concentrations of thrombin (0.05 to 0.08 unit), the antibody inhibited aggregation. Aggregation induced by the calcium ionophore A23187 (20 μ*M*) was not inhibited by UMR-304.

The specificity of UMR-304 for β_2m was confirmed by immunoprecipitation of ^{3}H-labeled HLA molecular complexes (*6*) from lymphoid cell extracts (Fig. 1). If UMR-304 was first reacted with β_2m, the ability of UMR-304 to precipitate HLA antigens was blocked. In similar experiments, intact platelets were labeled with ^{125}I and then reacted with UMR-304. Two peaks, a 44,000- and a 34,000-dalton species, were present in the immunoprecipitate. Both peaks were susceptible to immunodepletion with antisera to HLA framework antigens. The 12,000-dalton peak, but not the 34,000- or 44,000-dalton peaks, reacted with UMR-304 in electroblotting experiments. These results suggest that the 34,000-dalton peak was a proteolytic cleavage product of the HLA heavy chain. In another set of experiments, we tritium-labeled carbohydrate moieties of platelet membrane glycoproteins, either by enzymatic oxidation of galactosyl residues (*7*) or through Schiff-base formation by pyridoxal phosphate (*8*). With both techniques the 44,000- and 34,000-dalton peaks were present in SDS-PAGE gels after precipitation with UMR-304.

These results demonstrated that the principal platelet membrane antigenic species recognized by UMR-304 were elements of the HLA molecular complex. This suggested that the ability of UMR-304 to block the second wave of

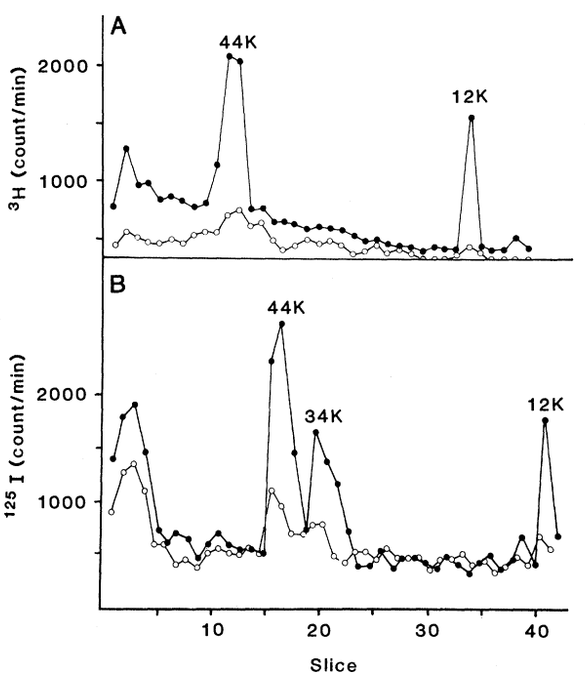

Fig. 1. (A) Immunoprecipitation of a biosynthetically labeled extract of the human lymphoblastoid cell line RPMI 1788 by the monoclonal antibody UMR-304. Cells were labeled with [^{3}H]leucine and extracted with 2 percent Non Idet P-40 in 50 m*M* tris buffer (NET buffer). The detergent extracts were centrifuged and the supernatants applied to *Lens culinaris* (lentil) lectin columns. After elution with α-methyl mannoside, samples were reacted with 20 to 40 μg of UMR-304 overnight at 4°C. The antigen-antibody complexes were adsorbed on protein A–Sepharose 4B beads. The beads were washed with NET buffer and the adsorbed proteins eluted in Laemmli sample buffer. Samples (20 μl) were applied to 10 percent Laemmli SDS-PAGE tube gels. After electrophoresis, β radioactivity of gel slices was determined. Peaks corresponding to the 44,000-dalton allotypic chain and the 12,000-dalton β_2m chain of the HLA molecular complex are evident in the precipitate (closed circles). Previous reaction of antibody with β_2m results in reduction of the characteristic HLA heavy and light chain signature (open circles). (B) Profile of ^{125}I-labeled platelet membrane proteins immunoprecipitated by UMR-304 as visualized on a 10 percent Laemmli SDS-PAGE gel. Two peaks of approximately 44,000 and 34,000 daltons are prominent; β_2m is found in the "spike" at the right end of the gel.

platelet aggregation was due either to a direct interaction of the monoclonal antibody with β_2m in the HLA molecular complex, or to interference with a closely adjacent surface membrane structure. It is known, however, that β_2m may associate with class I antigens other than HLA, such as Qa, Tl, and HY (*9*), and also that sequence homologies with β_2m occur in other immunologically important cell surface structures (*10*). It is possible that a minor platelet surface component, associated with or homologous to β_2m, but too low in concentration to be identified in the gels, could be the active site of the UMR-304 inhibitory effect.

To further define the antibody specificity involved, we produced four additional monoclonal antibodies to β_2m from BALB/c mice immunized with highly purified human β_2m. One of these antibodies, 14B006, inhibited human platelet aggregation in a manner similar to UMR-304. Two had no effect and one, 14B005, enhanced aggregation as has been previously described with polyclonal xenogeneic antibodies to β_2m (*11*). The different effects of these monoclonal antibodies are likely to relate to differences in their epitope specificity.

Since UMR-304 blocked the second wave of platelet aggregation, the possibility that UMR-304 inhibited formation of, or the platelet response to, endoperoxides or TXA_2 was evaluated. UMR-304 did not inhibit conversion of ^{14}C-labeled arachidonic acid to TXA_2, as measured by production of its stable metabolite thromboxane B_2 (*12*). However, UMR-304 uniformly inhibited the aggregation of gel-filtered human platelets induced 0.13 μM U46619, a potent, stable endoperoxide analog (*13*). The response of human platelets to TXA_2 was evaluated by a method (*14, 15*) that takes advantage of the fact that platelets obtained from all mongrel dogs form TXA_2 when stirred with sodium arachidonate, while platelets from only 40 percent of these dogs aggregate and secrete (*16*). C-PRP from dogs whose platelets do not aggregate or secrete when stirred with sodium arachidonate was used as a source of TXA_2. Addition of dog TXA_2 to gel-filtered human C-PRP, which had been incubated with ASA to block endogenous TXA_2 formation, resulted in prompt aggregation and secretion from the human platelets. Incubation of nonaggregating dog platelets with UMR-304 before stirring with sodium arachidonate did not inhibit TXA_2 formation since transfer of a portion of these platelets to human platelets resulted in normal aggregation. However, UMR-304 added to human platelets 1 minute before the addition of TXA_2 inhibited platelet aggregation and the secretion of adenosine triphosphate (ATP) (Fig. 2) and ^{14}C-labeled serotonin (data not shown). This inhibition was concentration-dependent. It varied somewhat in individual subjects, but $\geq$ 50 percent inhibition was achieved at UMR-304 concentrations of 2 to 10 μg/ml in all six subjects studied and 20 μg/ml uniformly abolished TXA_2-induced aggregation and secretion. The inhibitory effect of UMR-304 on TXA_2-induced platelet aggregation was identical to that of the endoperoxide-thromboxane receptor antagonist, 13-azaprostanoic acid (*17*). Neither 13-azaprostanoic acid (20 μM) nor UMR-304 (20 μg/ml) abolished TXA_2-induced platelet shape change.

The possibility that UMR-304 inhibited aggregation by inducing an elevation of platelet adenosine 3′,5′,-monophosphate (cyclic AMP) was considered. Incubation of gel-filtered human platelets with UMR-304 resulted in a slight but consistent elevation in cyclic AMP, measured by a protein binding assay (*18*). Mean concentrations of cyclic AMP were 19 percent higher in antibody-incubated platelets than in buffer-incubated platelets [8.46 ± 1.91 as opposed to 7.08 ± 1.75 pmole per milliliter of platelets; $P < 0.01$ by paired t-analysis; N = 28 (13 subjects)]. Prior incubation of platelets with 100 μM 2′,5′-dideoxyadenosine (DDA), an adenylate cyclase inhibitor (*19*), completely blocked inhibition of platelet aggregation by prostaglandin E_1 and prostacyclin; however, DDA had no effect on the inhibition of TXA_2-induced aggregation by UMR-304. Thus, the inhibitory effect of UMR-304 cannot be attributed to elevations of platelet cyclic AMP since: (i) UMR-304 did not inhibit the primary wave of platelet aggregation induced by ADP and epinephrine (*20*); (ii) the modest elevation in cyclic AMP observed was well below the magnitude of change reported to correlate with the degree of inhibition of platelet aggregation induced by UMR-304 (*19, 21*); and (iii) DDA did not block the platelet inhibitory effect of UMR-304.

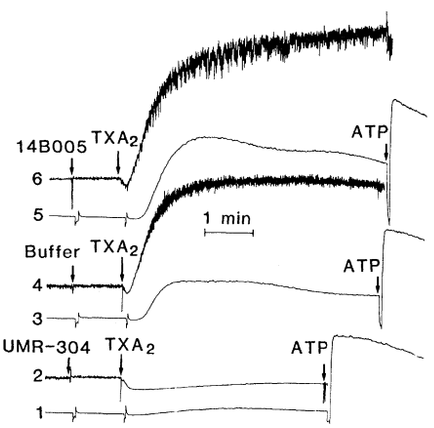

Fig. 2. Effect of adding UMR-304 to TXA_2-treated human platelets. C-PRP was prepared by the addition of 4.5 ml of blood to 0.5 ml of 3.8 percent sodium citrate followed by rapid mixing. C-PRP obtained from dogs whose platelets did not aggregate or secrete in response to sodium arachidonate (*15, 16*) was stirred at 100 rev/min with 0.3 mM sodium arachidonate in an aggregometer cuvette. Thirty seconds after addition of sodium arachidonate, a portion containing TXA_2 was removed for transfer to human platelets. C-PRP from normal human subjects was gel-filtered on Sepharose 2B columns, and the platelets were eluted with modified (*15*) Lindon's buffer (*28*). Platelets were then incubated with 0.1 mM acetylsalicylic acid (ASA) for 20 minutes at room temperature and stirred for 1 minute at 37°C in an aggregometer. Transfer of a 15 to 30 μl portion of TXA_2-containing dog C-PRP to 0.5 ml of ASA-incubated, gel-filtered human platelets resulted in prompt aggregation (curve 4) and release of adenine nucleotides (curve 3) measured as ATP in the Lumi-aggregometer (*29*). ATP ↑ indicates the point of addition of the ATP standard (1.2 μg). UMR-304 (20 μg/ml final concentration) added to human platelets 1 minute before addition of TXA_2 abolished aggregation (curve 2) and secretion (curve 1). Monoclonal antibody 14B005 to β_2m (20 μg/ml final concentration) augmented aggregation (curve 6) and secretion (curve 5).

These results suggest that UMR-304 blocks the endoperoxide-thromboxane receptor on the platelet membrane. Since UMR-304 binds to β_2m, it is possible that antibody binding to β_2m in the HLA complex inhibits access of endoperoxides and TXA_2 to their common receptor located in an adjacent site. Alternatively, β_2m or the HLA complex or both, may function as a receptor for endoperoxides and TXA_2, or play a role in processing the receptor or enhancing its signal. Recent evidence suggests that antibodies to HLA can block receptors for other mediators of cell function such as insulin, glucagon, γ-endorphin, and

epidermal growth factor (*24*). Other nonimmune functions proposed for molecules of the major histocompatibility complex (MHC) include binding of penicillin (*25*) and viruses (*26*) as well as participation in cellular adhesion and contact inhibition (*27*). If β_2m or the HLA complex are functionally related to the TXA_2 receptor, it is a new role for class I MHC molecules that will strengthen the concept that their function in cell biology is larger than that defined by their participation in immune responses.

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Ancient Bisexual Flowers

Abstract. *Fossil flowers discovered in 94-million-year-old clays of the Dakota Formation in Nebraska are among the earliest known demonstrably bisexual flowers. The flowers are of medium size and have pentamerous whorls of clearly differentiated floral parts, petals alternate with the sepals, short stamens are borne opposite the petals, the carpels are fused, and a receptacular disk is present. The pollen is small and tricolporate. These flowers appear to be well adapted to insect pollination. The numerous floral features and pollen characters provide sufficient diagnostic data to assess its systematic position. No extant order accommodates the features of this flower and it shares some features of various extant orders. The classification of flowering plants and our understanding of their evolution must be influenced by the fossilized remains of ancient flowers.*

Floral structure is the major basis on which living flowering plants of the world are classified (*1–3*). Orders, families, and lesser taxa can be characterized by certain common features of their reproductive organs. Pollen structure, wood anatomy, leaf morphology, and organic compounds have been used to supplement floral evidence in order to establish more "natural" groupings of major taxa (*3–5*) and to more clearly define relationships at the generic, specific, and subspecific levels.

The early fossil record of angiosperms (Barremian-Albian) consists mainly of pollen (*6–8*). Leaf remains are uncommon in deposits of this age but increase in abundance and complexity through the Aptian, Albian, and Cenomanian (*7–12*). A few early angiosperm reproductive remains have been reported (*8–17*).

We know from Tertiary sediments that fossil flowers do preserve and are of great importance in studying the evolution of flowering plants (*18*). However, there are very few detailed accounts of early angiosperm flowers from sediments of Cretaceous age (*14–17, 19*). Therefore, the discovery of one of the earliest known bisexual flowers with well-preserved floral parts is of particular interest. The collection of more than 50 whole or partial flowers preserved in different orientations and stages of development has allowed an accurate reconstruction of floral structure and reproductive biology (*20*). Remains of these flowers were discovered, collected, and studied as part of an ongoing project on the fossil plants of the Dakota Formation being conducted at Indiana University (*21*).

The flowers discussed in this report were collected at a locality in Nebraska from clays of the Dakota Formation (*22*). The Dakota Formation includes mainly nonmarine sandstones, shales, clays, and thin coals deposited along the shore and coastal plains of a mid-Cretaceous epicontinental sea which transgressed northward from the Gulf Coast area, eventually forming a connection with the Arctic Ocean. The beds from which the flowers were collected are dated at about 94 to 96 million years (*17, 23*) and are early Cenomanian (mid-Cretaceous) in age.

The flowers are pentamerous. Most frequently there remains only a thick receptacular disk with five sepals attached, forming a stellate pattern 2 to 3 cm in diameter (Fig. 1d). Ovate petals, 1 to 2 cm long, are preserved both isolated and occasionally attached to the receptacular disk. The five thin and delicate petals alternate with the robust sepals at the rim of the receptacular disk. Five stamens are attached to the receptacle, with one opposite each petal; the filament is flared at the base to form a broad connection to the receptacle (Fig. 1e). The stamens are 1 cm long, with filament and anther each being 0.5 cm long. The filaments are stout and the anthers are consistently four-locular and massive. The stamens preserved in place appear