titered inoculum ($10^{2.0}$ FFU) obviously replicated very poorly in the monkeys. Inocula containing similar concentrations of a DEN-2 (TH-36 isolate) temperature-sensitive-mutant produced detectable viremia in rhesus monkeys[4]. Indeed, such poor replication of DEN-3 has been observed in monkeys given much larger DEN-3 inocula (monkey No. 12, unpublished observations of this laboratory and S. B. Halstead et al.[9]). That limited replication of mutagenized virus occurred is inferred from the secondary-type antibody response showed following P_{23} challenge, since it is unlikely that the first inoculum (10^2 FFU) contained sufficient antigenic mass to sensitize the immune system by itself. This assumption seems reasonable in view of the slow primary-type antibody response observed in the control monkey inoculated with 10^7 mouse LD_{50} (representing a considerably larger antigenic mass). The primary-type response shown by this DEN-3-infected monkey is similar in temporal pattern to that shown by 17 seronegative rhesus monkeys inoculated with DEN-2 P_{23} mouse brain material or temperature-sensitive mutants[4]. The secondary-type response shown by the 2 monkeys given mutagenized virus and DEN-3 P_{23} materials resembles the temporal pattern observed in 14 monkeys challenged with DEN-2 P_{23} virus following infection with DEN-2 temperature-sensitive mutants[4]. Evidence has been presented that DEN-3 virus in minute quantities and without actually stimulating detectable antibody, is capable of sensitizing the immune system of rhesus monkeys to permit secondary-type antibody responses to subsequent DEN-3 infection. Whether this finding is applicable to other dengue virus types with greater reproductive capacity in human or primate hosts and to human immunological phenomena such as dengue hemorrhagic fever/shock syndrome is worthy of further study. The data also indicate the potential fallacy of inferring negative infection history from seronegativity.

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A peripheral high molecular weight glycoprotein located at the surface of human platelets

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Summary. A high molecular weight glycoprotein solubilised during platelet membrane isolation appeared to be a peripheral component of the human platelet surface and was susceptible to the action of neuraminidase when added to washed platelet suspensions.

Accumulating evidence suggests several possible roles for surface orientated oligosaccharides or their parent glycoproteins or glycolipids in platelet function[2–5]. It is therefore of interest to know more of the organization of the bound carbohydrate at the platelet surface. Following the homogenization of washed human platelet suspensions during the preparation of platelet membranes a prominent glycoprotein band of approximate molecular weight 148,000 has been located in the soluble cytoplasmic fraction by SDS-PAGE[6] analysis[4,7]. This glycoprotein (to be termed glycoprotein Is) is a constituent of the large glycoprotein band observed following SDS-PAGE of SDS and 2-mercaptoethanol solubilized washed human platelets and which is often called glycoprotein I[4,8]. Glycoprotein Is has been purified and characterized by Lombart et al.[7], is insoluble in low ionic strength solutions and contains approximately 60% carbohydrate and 13% sialic acid. As such it clearly differs from the 'thrombin sensitive protein'[9] which is an additional constituent of glycoprotein I of whole platelets[10]. The relationship between glycoprotein Is and a more firmly bound membrane glycoprotein of similar molecular weight[4,7] is unclear. A preliminary report has suggested that glycoprotein Is is located at the human platelet surface[11]. If this is so, then the bulk of its bound sialic acid would be susceptible to the action of neuraminidase when added to washed platelet suspensions.

Using a protease-free neuraminidase preparation of high specific activity (Vibrio cholerae, 3300 TBA units ml^{-1},

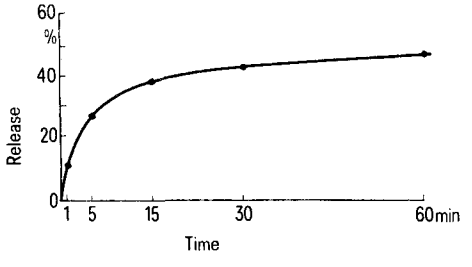

Fig. 1. Rate of release of sialic acid from washed human platelets by neuraminidase. Following washing (3 times) in 0.01 M tris HCl pH 7.4 containing 0.15 M NaCl, 1 mM EDTA and 0.35% bovine albumin, the platelets were finally resuspended (2×10^9 ml^{-1}) in 0.01 M tris HCl pH 7.0 containing 0.15 M NaCl, 2 mM $CaCl_2$, 0.35% bovine albumin and 200 µg ml^{-1} apyrase (Sigma). The incubation was performed at 37 °C with agitation in the presence of 50 units ml^{-1} neuraminidase. At increasing time intervals, aliquots were withdrawn, mixed with 4 mM EDTA to inhibit the enzyme, and the platelets sedimented at $3000 \times g$ for 20 min at 4 °C. Aliquots of the supernatants were assayed directly for free sialic acid[21], using a N-acetylneuraminic acid standard (Sigma Type IV). The results are expressed as the percentage of the total platelet sialic acid.

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Behringwerke Marburg, BRD)[12] conditions were established whereby approximately 40% of the total platelet sialic acid was released within 15 min of incubation at pH 7.0 (figure 1). Electrophoretic analysis (TGB-PAGE)[13] of the supernatant obtained following the sedimentation of the platelets revealed little sign of leakage of cytoplasmic proteins or release of lysosomal enzymes during the first 15 min of incubation with the neuraminidase. As platelet aggregation is inhibited at low pH[14], it was thought important to avoid the use of the acidic conditions often used during neuraminidase digestions and which may induce changes in surface architecture. It is highly probable that the rapidly released sialic acid is freely accessible to the neuraminidase on the washed platelet surface, an observation in agreement with the release of 60% of the total sialic acid of formalin-fixed human platelets by neuraminidase[15] and the reduced electrophoretic mobility of neuraminidase treated platelets[16].

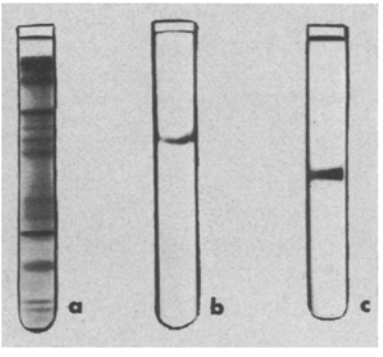

Fig. 2. The alcian blue staining capacity of glycoprotein Is. Washed human platelets were loaded with glycerol and osmotically lysed as described by Barber and Jamieson[18]. Following the sedimentation of all membrane material at 120,000 × g for 2 h at 4 °C, aliquots (200 µg protein, 0.1 ml) of the soluble cytoplasmic fraction were subjected to TGB-PAGE as previously described[17]. Protein (a) was located by amido black[22] and glycoprotein Is (b) by alcian blue at pH 2.5[19]. Following its exhaustive digestion with trypsin, glycoprotein Is gives rise to the faster migrating acidic macroglycopeptide (c), also located with alcian blue.

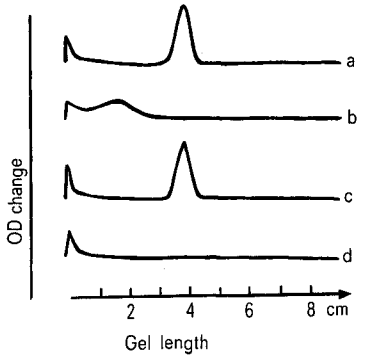

Fig. 3. Susceptibility of the acidic macroglycopeptide to neuraminidase digestion in washed platelet suspensions. Washed human platelets (2×10^9 ml^{-1}) were incubated in the presence (b, d) or absence (a, c) of neuraminidase (50 units ml^{-1}) as described in the legend to figure 1. The platelets were resuspended (2×10^9 ml^{-1}) in 0.01 M tris HCl pH 7.4 containing 0.15 M NaCl, 3 mM EDTA and 0.35% bovine albumin and incubated with trypsin (Sigma, Type I) for 1 h at 37 °C as previously described[17]. Aliquots (0.2 ml) of the supernatant were subjected to TGB-PAGE[17], the gels fixed for 1 h in 12.5% w/v trichloroacetic acid, washed in 2% acetic acid and stained by the periodate-Schiff reaction[23] (a, b) or by alcian blue pH 2.5 (c, d). The gels were densitometrically scanned at 546 nm.

Glycoprotein Is has been shown to be a precursor of a trypsin-resistant acidic macroglycopeptide of molecular weight 120,000[7] which has been suggested to have a characteristic staining capacity with the cationic dye alcian blue at pH 2.5, the alcian blue binding to neuraminidase sensitive sialic acid groupings[17]. Following its solubilization during platelet lysis, glycoprotein Is migrates as a beta-globulin on TGB-PAGE (figure 2). The identification of this band was confirmed by its comigration with glycoprotein Is purified as described by Lombart et al.[7]. When neuraminidase treated platelets which had lost 40% of their sialic acid were homogenized during the glycerol lysis procedure[18], the released glycoprotein Is no longer stained with alcian blue and showed a reduced staining with the periodate-Schiff reaction. When the neuraminidase treated platelets were digested with trypsin under conditions which normally give the maximal release of bound sialic acid and macroglycopeptide[17,19], the released macroglycopeptide did not stain with alcian blue, migrated more slowly on TGB-PAGE and showed a 60% reduction in its staining capacity with the periodate-Schiff reaction (figure 3). Essentially similar results to these for both alcian blue and periodate-Schiff staining were obtained when glycoprotein Is purified according to Lombart et al.[7] and the acidic macroglycopeptide obtained following the 30 min digestion of washed platelet suspensions[17] were incubated with neuraminidase under the same conditions as described for the neuraminidase digestion of the washed platelet suspensions. Furthermore SDS-PAGE of solubilised whole platelets indicated an apparently normal release of glycoprotein Is during the trypsin digestion of neuraminidase treated platelets. The evidence suggests, therefore, that glycoprotein Is is susceptible to neuraminidase digestion in washed platelet suspensions at pH 7.0 and is a probable component of the platelet surface under physiological conditions.

Previous studies have shown that an alcian blue staining acidic glycopeptide of apparent molecular weight 147,000 was released during the early stages of trypsin digestion, and that this released glycopeptide subsequently gave rise to the macroglycopeptide[17,19]. The 147,000 molecular weight acidic glycopeptide obtained following a 10 sec trypsin digestion of washed human platelets[19] comigrates with glycoprotein Is on TGB-PAGE. Thus part, at least, of glycoprotein Is may be released having undergone little or no proteolysis. As trypsin is unlikely to induce disruption of the membrane lipids, it would appear that glycoprotein Is is not an intrinsic membrane component as defined by Singer[20], but is a peripheral component loosely attached to the platelet surface.

Note added in proof: Glycoprotein Is has recently been termed 'glycocalicin' by Okumura and Jamieson[24].

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