Immunological sensitization following inapparent infection with dengue virus type 3 in rhesus monkeys

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Summary. Rhesus monkeys previously given dengue virus type 3, without apparent viremia or antibody response, exhibited a secondary-type response upon reinoculation. These data suggest that monkeys can be immunologically sensitized to dengue virus without detectable antibody production.

Recently, we reported that temperature-sensitive mutants of dengue virus type 2 (DEN-2) could be induced chemically with 5-azacytidine, and that some of these had diminished ability to produce viremia while still inducing a significant immune response in rhesus monkeys[3,4]. We also attempted similar studies with DEN-3. However, the generally inferior reproductive capacity of DEN-3 in diploid mammalian cells frustrated most of these attempts. We did observe and now report an interesting phenomenon during the course of that work, which we believe may represent the first known demonstration of immunological sensitization to live dengue virus in primates without concommitant detectable antibody synthesis under controlled laboratory conditions.

Materials and methods. Primary hamster kidney (HK) cell culture was prepared as reported previously[5]. LLC-MK_2 cells were maintained as previously described[3]. DEN-3 (H87 prototype strain) at its third blind mouse brain passage (P_3) did not replicate or form fluorescent foci[6] in 4 diploid primate cell culture systems tested. However, the virus replicated and formed antigen in HK cells. After 4 serial passages in HK cells at 37 °C, the virus (designated P_3T_4) was incubated at 37 °C for 20 min in 100 µg/ml N-methyl-N'-nitro-N-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.). This material was cloned at 33.5 °C by fluorescent antibody technique reported earlier[6]. Of 12 clones originally isolated, only one clone could be propogated at 33.5 °C in HK cells. After 2 passages, this material was found to contain $10^{2.0}$ fluorescent focus-forming units (FFU)/0.1 ml at 33.5 °C, and less than $10^{1.0}$ FFU/0.1 ml at 40 °C, by previously described methods[3]. DEN-3 H87 virus in its 23rd suckling mouse brain passage was propogated and titrated as described by Hammon and Sather[7]. This material contained $10^{7.0}$ suckling mouse LD_{50}/0.1 ml, and formed essentially equal numbers of fluorescent foci in HK cells at 33.5 °C and 40 °C.

Juvenile 4–6 lb female rhesus monkeys were obtained from Primate Imports Corp., Port Washington, N. Y. Animals were maintained as described previously[4]. Animals were free of tuberculosis by skin test throughout the experimental period. As reported previously[4], all animals were screened for dengue and other flavivirus antibodies before use. Hemagglutination-inhibition (HI) tests were performed as described by Hammon and Sather[7].

Outbred suckling HaM/ICR mice (1–2 days old) were obtained from Charles River Breeding Laboratories, Wilmington, Mass., and maintained as previously described in detail[8].

Results and discussion. DEN-3 virus exposed to N-methyl-N'-nitro-N-nitrosoguanidine replicated so poorly that it is not certain whether it represents a temperature-sensitive mutant. It can only be described as a virus which produced detectable antigens at 33 °C but not at 40 °C.

On day 0, 2 seronegative monkeys (No. 6 and 7) were inoculated s.c. in the right forearm with 0.1 ml of the HK cell-propagated mutagenized virus (P_3T_7). Sera were obtained on days 3 through 7, 14 and 21. All attempts to isolate DEN-3 by intracerebral inoculation of sera into suckling mice, or by inoculation in HK or LLC-MK_2 cells were unsuccessful. Sera were also negative for DEN-3 HI antibody (table).

On day 52, these 2 monkeys plus a previously uninfected monkey (No. 12) were inoculated s. c. in the left forearm 0.1 ml DEN-3 H87 P_{23} virus. Serial bleedings on days 55 through 59 again failed to contain detectable virus. Antibody levels rapidly rose in the 2 monkeys previously given mutagenized virus, while in the control monkey, HI antibody was not detected until 14 days following challenge; and this at a considerably lower titer (table). These findings are suggestive of immunological sensitization without detectable antibody production in the 2 monkeys receiving the mutagenized virus. The low-

Serum HI antibody titers to DEN-3 prior and subsequent to vaccination and challenge*

Monkey No.	HI titer on day −7	0	7	14	21	52	59	66
6	<10	<10	<10	<10	<10	<10	160	320
7	<10	<10	<10	<10	<10	<10	160	40
12	<10	ND**	ND	ND	ND	<10	<10	10

* Monkey 6 and 7 were vaccinated with P_3T_7 on day 0; all 3 monkeys received P_{23} mouse brain material on day 52. ** Not done.

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2 The authors wish to express their deepest gratitude to Alma Till man, Carlie White, Annie McCoy, Francis Brisset, Albert Jackson and Becky Kagarise for their devoted technical assistance. This research was supported in part by the U.S. Army Medical Research and Development Command under Contract No. DADA-16-69-C-9048 and DAMD-17-74-C-4005 and by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U.S. Public Health Service, under Research Grant No. 5-R22-AI02686-13, -14 and -15, and Training Grant No. 5-T01-AI-00110-14.

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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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9 August 1976

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},

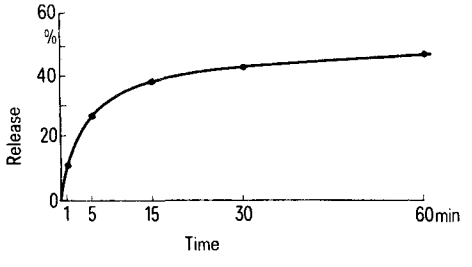

Fig. 1. Rate of release of sialic acid from washed human platelets by neuraminidase. Following washing (3 times) in 0.01 M tris HCl pH 7.4 containing 0.15 M NaCl, 1 mM EDTA and 0.35% bovine albumin, the platelets were finally resuspended (2×10^9 ml^{-1}) in 0.01 M tris HCl pH 7.0 containing 0.15 M NaCl, 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.

1 Part of this work was performed at The Nuffield Institute of Comparative Medicine, Zoological Society of London, Regents Park, London (England).

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