Immunological enhancement and the pathogenesis of dengue haemorrhagic fever

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SUMMARY

Laboratory studies have provided evidence that the replication of dengue viruses in preparations of primary peripheral blood mononuclear cells of human or simian origin, or in macrophage-like cell lines of human or murine origin, may be enhanced by sub-neutralizing concentrations of homotypic dengue antibody, by heterotypic dengue antibody, or by antibody against heterologous flaviviruses. The mechanism underlying this phenomenon is discussed in the context of dengue haemorrhagic fever and the dengue shock syndrome.

The concept that antiviral antibodies might potentiate viral infection seems nonsensical, but evidence is accumulating that this is indeed the case in dengue haemorrhagic fever (DHF) and the dengue shock syndrome (DSS), conditions that are recognised to be major causes of morbidity and mortality in children in South East Asia, and more recently in the Caribbean region also (Halstead, 1980a, WHO, 1981). The key to the enigma may lie in the fact that not one virus, but four antigenically related viruses are involved. The outcome of infection with any one of the four serotypes of dengue virus depends critically upon the immune status of the individual at the time of exposure. Infection in a non-immune subject results only in a benign, self-limiting febrile illness, usually associated with a rash; this is classical dengue fever, which may involve painful joint symptoms (break-bone fever), but is virtually never fatal (Siler, Hall & Hitchens, 1926; Schlesinger, 1977). Infection in an individual with antibodies depends upon the specificity of the relationship between those antibodies and the infecting dengue virus. Antibodies against any one serotype are protective against reinfection with that particular serotype, but confer no heterologous cross-protection (Sabin, 1952). On the contrary, heterotypic antibody may complex with virus and potentiate the infection of macrophages, with consequent increased yields of virus and increased disease.

This considerably over-simplified picture of the pathogenesis of DHF and DSS has been arrived at after careful epidemiological studies of patients suffering from various forms of the disease, after laboratory studies utilising rhesus monkeys and other animals, followed by observations on the enhanced replication of dengue virus in peripheral blood mononuclear cells. Some knowledge of the molecular

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biology of dengue virus is necessary for an understanding of the specificity of the antibody mediated enhancement of viral replication.

Taxonomically, dengue viruses are placed within the genus flavivirus (named after the type species, yellow fever virus) in the family Togaviridae. There are some 60 different viruses in the genus, about one half of which are transmitted by mosquitoes (as is the case with dengue viruses), about one quarter are tick transmitted, and the remainder appear to spread without an arthropod vector. All 60 members are believed to share the basic common features of having a single molecule of linear, single stranded RNA, together with three viral proteins, a nucleocapsid protein, N, and a core protein, C, (both of which are non-glycosylated), and a single glycosylated envelope protein, E. Individual flaviviruses show different degrees of antigenic inter-relationship depending upon the serological test being used. Thus there is very extensive cross-reactivity by haemagglutination inhibition (HI) tests, but almost complete specificity by neutralisation tests (NT), with intermediate cross-reactivity detected by complement fixation (CF) tests. The antigenic specificity of different flaviviruses is primarily dependent upon the envelope glycoprotein, E, which is known to have determinants which confer group specific, sub-group specific, and type (or virus) specific reactivity (Trent, 1977).

Epidemiological evidence

Classical dengue fever can occur at any age, but the more severe forms of dengue infection, DHF and DSS, are seen almost exclusively in children, where a bimodal age distribution is apparent. The first peak is seen in infants, and the second in children aged 3–4, but sometimes later than this. DHF and DSS occur principally in large urban centres, where there is extensive mosquito breeding, notably of *Aedes aegypti* (which is also the main vector of urban yellow fever). In cities such as Bangkok, where all four dengue virus serotypes circulate freely, virtually every adult will have antibodies against all four types, and children born of such mothers will initially be protected by maternal antibodies. However, as levels of maternally derived antibodies decline, infants are increasingly at risk of contracting DHF or DSS; these cases account for the first peak in the age distribution curve. Such infections are 'primary', in that they represent the particular infant's first exposure to dengue virus, but they occur in infants 'sensitized' by antibodies which have fallen below the protective threshold but are still capable of 'enhancing' viral replication. If exposure is delayed beyond about 1 year, maternal antibodies will neither protect nor sensitize, and the outcome of these 'primary' infections will be represented by the development of classical dengue fever only. In subsequent seasons, children who have experienced benign, primary infections will be at risk of developing DHF or DSS if they encounter infection with a different dengue virus serotype. The children in this set comprise the second of the two peaks in the age distribution curve. For reasons which are not clear, there is a preponderance of severe cases in girls.

Immunological enhancement in vivo

There is no really satisfactory experimental animal model for haemorrhagic dengue, but studies in monkeys have provided valuable clues as to what may happen in human dengue infections. Levels of viraemia detected on successive days after inoculation with dengue virus were found to be significantly higher in

monkeys which had heterotypic dengue virus antibodies than in those completely without dengue antibodies (Halstead, Chow & Marchette, 1973). Furthermore, when heterotypic antibody was passively administered to non-immune rhesus monkeys 15 min before dengue virus was injected, viraemia levels were significantly higher than those seen in monkeys given normal serum prior to the same viral challenge (Halstead, 1979).

Immunological enhancement in vitro

Peripheral blood mononuclear cells prepared from dengue immune monkeys or humans support the replication of dengue virus, whereas similar cells prepared from non-immune donors are essentially non-permissive (Halstead *et al.* 1973, Marchette, Halstead & Chow, 1976, Halstead *et al.* 1976). When non-permissive human or monkey peripheral blood mononuclear cells are exposed to dengue virus in the presence of passively added anti-dengue antibody, viral replication takes place, provided that the antibody concentration is below the level that would neutralize the virus (Halstead & O'Rourke, 1977). These experiments provide the base upon which all subsequent dengue virus enhancement studies have been founded. Although the phenomenon was reproducible, the magnitude of the enhancement varied from one experiment to another, even when the same donor was used repeatedly. This inherent variability associated with the use of primary preparation of mononuclear cells raised the possibility that more reproducible results might be obtained by the use of continuous lines of cells having macrophage-like properties.

Mechanism of immunological enhancement of viral replication

The three components which interact in antibody mediated enhancement of viral replication are the virus, the host cells, and antiviral antibodies. Using dengue virus type 2, Halstead & O'Rourke (1977) demonstrated a dependence upon cellular Fc receptors, those on the human mononuclear cells they were using being trypsin resistant. They also showed that enhancement was mediated by antibodies of the IgG class, that IgM and F (ab')$_2$ fragments were not able to enhance (although IgM showed weak activity in the presence of complement, Halstead, 1980*b*), and that the mammalian species in which the antibodies were raised was immaterial (Halstead & O'Rourke, 1977). The trypsin sensitivity of the dengue virus receptors on human mononuclear cells has been further studied by Daughaday *et al.* (1981). They showed that aggregated gamma globulin would block the enhancement produced by dengue virus complexed with dilute anti-dengue antibodies. They also showed that although F (ab')$_2$ fragments prepared from dengue immune human serum were without enhancing activity, the addition of rabbit anti-human Fab to these F (ab')$_2$ fragments restored enhancing activity when tested on trypsin treated human monocytes, apparently through the Fc portion of the rabbit anti-Fab IgG attaching to Fc receptors on the monocytes.

Immunological enhancement in macrophage cell lines

Several continuous lines of cells that have macrophage-like morphology and express a number of markers of macrophage function are now recognized (Morahan, 1980). Although differing in some respects from primary macrophages, these cell lines, here referred to as macrophage cell lines, offer potential advantages for the

study of immunological enhancement by virtue of their well defined characteristics and the reproducibility of their properties upon serial cultivation. Two macrophage cell lines of mouse origin, P388D1 and J774.1, and the U937 line of human origin, were tested by Peiris & Porterfield (1979); they failed to detect replication of dengue virus in these cells, but were able to show that another flavivirus, West Nile virus (WNV), did replicate in all three cell lines, and that virus yields were enhanced in all three lines by appropriate concentrations of antiviral antibody. Enhancement could be detected by a two stage assay, using plaque formation in the PS line of pig kidney cells to titrate the yields of WNV produced in macrophages infected either with or without antiviral antibody. Alternatively the PS cells could be used to measure the number of infectious centres induced in macrophages infected under the different conditions. However, a direct assay of antibody dependent plaque enhancement (ADPE) was possible in one of the mouse macrophage cell lines, since lytic plaques formed in P388D1 monolayers when these were incubated under carboxymethyl cellulose overlays (Peiris & Porterfield, 1979, 1981). This direct ADPE assay was not possible with either the J774.1 or U937 cells, since these attach very poorly to glass or plastic surfaces. Recently, direct ADPE tests have been performed with WNV and another mouse macrophage cell line, NCTC 1469, (Porterfield, unpublished observations). Some of the properties of four macrophage cell lines are presented in Table 1.

Immunological enhancement in P388D1 cells

Having demonstrated that the phenomenon of antibody mediated enhancement of viral replication first described by Halstead and his colleagues in the context of dengue virus enhancement in primary human or monkey mononuclear cells could be reproduced in macrophage cell lines, albeit with a different flavivirus, Peiris *et al.* (1981 *a, b*) next explored the mechanism underlying the phenomenon. Two distinct Fc receptors have been demonstrated on mouse macrophage cell lines, FcRI, which is trypsin sensitive and binds IgG2a, and FcRII, which is trypsin resistant and binds IgG2b and IgG1 complexes (Unkeless, 1977, Haeffner-Cavaillon, Klein & Dorrington, 1979, Diamond, Bloom & Scharff, 1980). A monoclonal antibody which binds specifically to FcRII receptors on P388D1 and J774.1 cells has been prepared (Unkeless, 1979). When this monoclonal antibody was applied to P388D1 cells which were infected either with WNV alone or WNV complexed with anti-WNV antibody, antibody mediated enhancement of viral replication was blocked (Peiris *et al.* 1981 *a, b*). A second monoclonal antibody directed against a different, and irrelevant, mouse macrophage surface antigen, F4/80 (Austyn & Gordon, 1981) had no such blocking activity.

Further evidence that immunological enhancement of viral replication is dependent upon Fc receptors has come from studies using hybrid cell lines, derived from a mouse melanoma cell line (PG19) fused with mouse peritoneal macrophages. One such hybrid cell line, D2–7, expresses Fc receptors, whereas a second cell line, C1, does not. West Nile virus replicates in all three lines, although less well in the macrophage cell line, D2–7, than the others. However in the presence of dilute anti-WNV antibody, yields of WNV were enhanced about 40 fold in D2–7 cells, whereas yields in the parent cell line and the C1 cells, which lack Fc receptors, remained unchanged. (Peiris *et al.* 1981*a*).

Table 1. *Continuous cell lines having macrophage-like properties*

Cell line	P388D1	J.774.1	NCTC 1694	U937
Origin	DBA/2 mouse	Balb/c mouse	C3H mouse	Human
Derivation	Methylcholanthrene induced tumor	Oil induced tumour	Liver cells	Histiocytic lymphoma
Fc receptors	+	+	+	+
Complement receptors	+	+	+	+
Phagocytic	+	+	+	+
Reference	Koren, Handwerger & Wunderlich, 1975	Ralph, Prichard & Cohn, 1975	Evans *et al*. 1952 Van Loveren *et al*. 1981	Sundstrom & Nilsson, 1976

Using P388D1 cells, Peiris & Porterfield (1981) were able to demonstrate antibody dependent plaque enhancement with six flaviviruses, (two strains of West Nile, Kunjin, Murray Valley encephalitis and Uganda S viruses), three alphaviruses, (Semliki Forest, Sindbis and Western Equine encephalitis viruses), three Bunyaviridae, (Bunyamwera, Lokern and Trivittatus viruses), but were unable to show enhancement with Herpes simplex virus (family Herpesviridae) or with Mengo virus (family Picornaviridae). Although negative results were initially reported (Peiris & Porterfield, 1979) more recent tests with dengue virus, using the 16681 strain, have succeeded in demonstrating enhancement, although without any cytopathic effect. (Porterfield *et al*. 1982).

Immunological enhancement in U937 cells

Enhanced replication in the presence of antiviral antibody has been reported to occur in U937 cells with three different flaviviruses, West Nile virus, (Peiris & Porterfield, 1979), yellow fever virus, (Schlesinger & Brandriss, 1981), and dengue virus, (Brandt *et al*. 1982). The magnitude of the West Nile virus enhancement was similar to that observed in P388D1 cells, and since the latter cells permitted a direct estimate of enhancement by counting plaque numbers, Peiris & Porterfield (1979, 1981) did most of their work on P388D1 cells. Schlesinger & Brandriss (1981) reported that U937 cells were permissive for the replication of yellow fever virus but no cytopathic effect was produced. Washed U937 cells were infectd with 17D yellow fever virus in the presence or absence of yellow fever antibodies for 1 h either at 4 or 37 °C; the cells were then washed free from residual virus and antibody and incubated in antibody free medium for 48 h at 37 °C, at which time virus yields were titrated in BHK-21 cells. Substantially more virus was produced from cells infected at 37 °C than from those infected in the cold, but yields were increased only slightly and irregularly by the presence of antibody in the former possibly because some neutralization took place under these conditions, but were enhanced 7–57 fold by antibody when infection occurred at 4 °C. Trypsin treatment of U937 cells resulted in a 90–95 % reduction of yields of yellow fever virus, but trypsin treated cells which were infected in the presence of antibody gave increased yields as compared with untreated U937 cells also infected in the presence of antibody. Yellow fever virus infection of U937 cells appears not to be a very efficient system, in that no more than 0·3 % of cells became productively infected, even in the presence of antibody, and the percentage was much lower without antibody (Schlesinger & Brandriss, 1981). These authors reported that virus yields varied

considerably and unpredictably in the different experiments carried out under apparently identical conditions.

Specificity of immunological enhancement

If immunological enhancement of viral replication contributes to the pathogenesis of DHF and DSS, it becomes a matter of major importance to determine the precise antigenic specificity or specificities involved in triggering this reaction. Using dengue virus type 2 as the test virus, Halstead & O'Rourke (1977) showed that antisera against heterotypic types 1, 3 & 4 dengue viruses would all enhance, as would homotypic antibody when diluted beyond the neutralising endpoint. In a separate study, again with dengue virus type 2, and using human mononuclear cells, Halstead, Porterfield & O'Rourke (1980) showed that a wide range of antisera prepared in rabbits against different flaviviruses were capable of mediating enhancement; antisera prepared in rabbits against alphaviruses, Bunyaviridae, or against normal mouse brain had no enhancing activity.

A major difficulty in interpreting the significance of these observations is that the antibodies used were all post-infection or post-immunization sera which would inevitably contain antibodies against a variety of different viral antigens. The advent of monoclonal antibodies has opened up new possibilities for exploring the enhancement phenomenon, and evidence from this type of approach is now beginning to appear.

Using the West Nile virus model, Peiris, Porterfield & Roehrig (1982) reported that monoclonal antibodies with three different specificities all enhanced the yields of WNV as measured by plaque formation in P388D1 cells. All three were reactive with WNV envelope glycoprotein in radioimmune precipitation tests, but only one, F6/16A was able to neutralise WNV well. The other two monoclonal antibodies, F7/3 and F7/101, had virtually no neutralizing capacity when tested separately, but when combined they had moderate neutralizing activity. Antibody F7/3 had strong HI activity, not only against WNV, but also against other flaviviruses, including dengue virus, and probably represents an antibody with group reactive specificity. Antibody F6/16A has narrow type or strain specificity, being able to distinguish between the homologous Egypt 101 strain of WNV, which it neutralizes, and the Smithburn strain of WNV, which is not neutralized. The third monoclonal antibody, F7/101, has sub-group specificity, being able to enhance the replication of both strains of WNV, and of the closely related Kunjin virus, but this antibody was unable to enhance the replication of the more distantly related Uganda S virus.

Although no monoclonal antibodies with specificities against the viral nucleocapsid or core were available, the West Nile findings strongly implicate antibodies directed against the viral envelope as being the relevant antibodies in the enhancement phenomenon.

Two groups have now reported the availability of monoclonal antibodies against dengue viruses. Dittmar, Haines & Castro (1980) reported the preparation of monoclonal antibodies against dengue virus type 3. Several antibodies were reactive by HI and by immunofluorescence with all four dengue serotypes, but one cloned antibody appeared to be specific for type 3 virus only. The biological activities of these monoclonal antibodies were not reported. Gentry et al. (1982)

have prepared monoclonal antibodies against dengue virus type 2, and some of these antibodies have been used in a study of enhancement in the human macrophage cell line U937 (Brandt *et al.* 1982). None of five different monoclonal antibodies which showed type specific activity by HI, NT and IF, was able to enhance the replication of dengue virus type 2. In marked contrast, all of seven monoclonal antibodies which showed broad flavivirus cross-reactivity by HI were also able to enhance the replication of dengue 2 virus in U937 cells; most of these antibodies showed some cross reactions with other dengue virus serotypes by NT also.

Enhancing antibodies in naturally acquired dengue virus infections

We have recently been examining a number of human sera collected from individuals who have had dengue virus infections of varying degrees of severity, and have measured enhancing antibodies against both West Nile and dengue viruses, as well as antibodies detected by more conventional means, such as by haemagglutination inhibition and neutralisation tests. (We are indebted to Dr D. W. Burke for many of these sera, and for details of serological tests carried out in Bangkok). Sera from six Thai children who have had primary dengue virus infections, determined on the basis of their HI patterns in paired specimens, showed no enhancing activity in the acute phase serum against either WNV or dengue virus type 2 viruses, when the sera were tested over the range of serum dilutions from 10^{-1} to 10^{-6}. Convalescent sera from all six subjects enhanced WNV at 10^{-2} to 10^{-3} serum dilutions, and all six enhanced dengue virus at dilutions from 10^{-4} to 10^{-6}. When sera from six Thai children who had had secondary dengue virus infections were examined in the same way, acute and convalescent sera from all six enhanced both viruses. Three pairs of sera showed rising titres against dengue virus type 2 and two of these three also showed rising titres against WNV. All six convalescent sera were active in enhancing dengue virus at 10^{-6} serum dilution (Porterfield *et al.* 1982).

The immunopathological mechanism described above provides a means whereby antiviral antibody can promote enhanced dengue virus replication, both *in vitro* and *in vivo*, even if some details of the precise specificity of the reaction remains to be clarified. There are, however, many aspects of the pathogenesis of DHF and DSS that remain obscure. It has been estimated that only about 3 % of children with dengue antibodies who are at risk of DHF or DSS actually develop severe disease (Halstead, 1980b). The factors that determine the outcome in any particular infection are largely unknown. It has been suggested that there might be some histocompatibility linked susceptibility to dengue virus infection, but so far the evidence for this is slight (Chiewsilp, Scott & Bhamarapravati, 1981).

The problems of attempting to vaccinate against the severe forms of dengue virus infection are formidable. Since heterotypic antibodies may potentiate infection, immunization against all four serotypes would seem to be the only safe course. There are, unfortunately, very real difficulties in devising polyvalent vaccines that would confer satisfactory immunity against each of the component types. A number of experimental vaccines are under development or trial, but the prospect of a safe, potent, polyvalent vaccine that could be used in children and that would be effective in preventing DHF and DSS is remote. The recent extension of

haemorrhagic dengue to the Caribbean region emphasizes the need for further research in this medically important and scientifically intriguing problem.

Although most of the published evidence points to Fc receptors as the relevant cellular binding sites through which virus-antibody complexes attach to host cells, the possibility remains that other cellular receptors might function in a similar way. Complement receptors are present on many different cell types, and like Fc receptors they have several different specificities. Some recent work in Oxford has shown that IgM and complement receptors are capable of mediating enhanced dengue virus infection of primary macrophage preparations under laboratory conditions (Cardosa, M. J., unpublished observations). This pathway may explain the rare instances of haemorrhagic dengue in primary infections when the role of maternally derived antibodies can be excluded.

Although this paper has been concerned principally with the pathogenesis of haemorrhagic dengue and the dengue shock syndrome, it is worth noting that parallel aberrant virus-antibody interactions may operate in such conditions as the early death syndrome in rabies, and in the severe respiratory symptoms that appear in infants who have received vaccine against respiratory syncytial virus and are subsequently exposed to natural infections with this virus. Beyond the field of viral diseases, some of the problems associated with attempted immunization against chlamydiae and mycoplasmas may possibly reflect antibody mediated enhancement of infection following subsequent exposure. The host-immune mechanisms are highly complicated, and it is perhaps not altogether unexpected that these sometimes operate to the disadvantage of the host.

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