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Antibody-dependent Enhancement of Plaque Formation on Cell Lines of Macrophage Origin – A Sensitive Assay for Antiviral Antibody

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SUMMARY

An assay for antiviral antibody based on antibody-dependent plaque enhancement (ADPE) in the macrophage cell line P388D1 is described which is as sensitive as, or more sensitive than a radioimmune assay. The method is applicable to a range of Togaviridae and Bunyaviridae although not to Picornaviridae and Herpesviridae. The variables affecting the assay are investigated.

INTRODUCTION

The replication of Togaviridae in macrophage cell lines (P388D1; U937) can be enhanced 100-fold in the presence of low concentrations of specific antiviral antibody (Peiris & Porterfield, 1979). In P388D1 cells, this enhancement can be blocked by monoclonal antibody with specificity against the Fc receptor, demonstrating that the phenomenon depends on the presence of the Fc receptor (Peiris *et al.*, 1981 *a*, *b*). In this paper we describe a simple method for demonstrating antibody-dependent plaque enhancement (ADPE) in P388D1 cells, and show that this is a highly sensitive assay for antiviral antibody, as sensitive as radioimmune assay. The variables affecting the assay are investigated and experiments with viruses in four families, Togaviridae, Bunyaviridae, Herpesviridae and Picornaviridae are presented.

METHODS

Cells. The P388D1 macrophage cell line (Ralph, 1980; Koren *et al.*, 1975) was grown in 75 cm^2 Falcon flasks (Becton Dickinson, Oxnard, U.S.A.) in Alpha modification of minimum essential medium (Gibco-Biocult, Glasgow, U.K.) (MEM-α), supplemented with 10% heat-inactivated (56 °C, 30 min) foetal calf serum (HIFCS) (Sera Lab., Crawley, Sussex, U.K.) and antibiotics (complete MEM-α). When confluent, the P388D1 cells were detached by vigorous shaking. (These cells are not removed by conventional trypsin–EDTA treatment.)

Virus stocks and antisera. The viruses used and their strain documentations are given in Table 1. All were prepared as 10% infected suckling mouse brain homogenates (Shope & Sather, 1979) except herpes simplex type 1 virus which was a freeze-thawed suspension of infected Vero cells. Virus preparations were clarified by centrifugation at 500 ***g*** for 1 h at 4 °C and stored at −70 °C.

Rabbit antisera to Togaviridae, Bunyaviridae and Picornaviridae were prepared as described by Madrid & Porterfield (1974). Hyperimmune mouse ascitic fluids to West Nile virus (WNV) were prepared as described by Shope & Sather (1979), mice being immunized first with β-propiolactone-inactivated virus in Freund's complete adjuvant injected subcutaneously, followed by 2 to 6 doses of live virus given intraperitoneally at 3 weekly intervals. Ascites were induced with Ehrlich ascites tumour cells. Antiserum to herpes simplex

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Table 1. *Viruses studied for plaque formation and ADPE on P388D1 cells*

Virus	Strain	Passage level	Virus stock prepared as	Time for plaque formation in P388D1 cells (days)	ADPE demonstrable
Flaviviruses					
West Nile	Egypt 101	5	10% Mouse brain	3–4	Yes
West Nile	Smithburn	NK*	10% Mouse brain	3–4	Yes
Alfuy	MRM3929	7	10% Mouse brain	NPF*	–
Dengue type II	Tr 1751	56	10% Mouse brain	NPF	–
Israel Turkey	Original	31	10% Mouse brain	NPF	–
Kunjin	MRM16	8	10% Mouse brain	3	Yes
Murray Valley encephalitis	1/1951	16	10% Mouse brain	5	Yes
Tembusu	M1775	12	10% Mouse brain	NPF	–
Uganda-S	Original	30	10% Mouse brain	5	Yes
Alphaviruses					
Semliki Forest	Smithburn	NK	10% Mouse brain	3	Yes
Sindbis	EgAr 339	NK	10% Mouse brain	3	Yes
Western equine encephalitis	Rockefeller	NK	10% Mouse brain	3	Yes
Bunyaviridae					
Bunyamwera	Smithburn	12	10% Mouse brain	4	Yes
Lokern	FMS 4332	14	10% Mouse brain	4	Yes
Trivittatus	L30406	10	10% Mouse brain	5	Yes
Herpesviridae					
Herpes simplex type 1		NK	Vero cell extracts	4	No
Picornaviridae					
Mengo	Original	NK	10% Mouse brain	3	No

* NK, Not known; NPF, no plaque formation.

type 1 virus was kindly provided by Dr R. W. Honess (National Institute for Medical Research, Mill Hill, London U.K.). This had been prepared by immunizing a rabbit with herpes simplex type 1 virus-infected rabbit kidney cell culture material.

Overlay media and stains. Carboxymethylcellulose (CMC) (sodium salt, low viscosity; BDH Chemicals) overlay in MEM-α or L-15 medium was prepared by mixing equal volumes of 3% CMC in deionized water (sterilized by autoclaving) and double-strength medium supplemented with HIFCS.

Naphthalene black (G. T. Gurr, Colour Index 20470) stain was made up to 0·1% (w/v) in distilled water containing 6% (v/v) glacial acetic acid and 1·36% (w/v) sodium acetate.

Assays for antiviral antibody. Haemagglutination inhibition (HI) and 50% plaque reduction neutralization tests (PRNT) on PS cloned cells were done as described by Shope & Sather (1979) and Madrid & Porterfield (1969) respectively. Solid-phase radioimmune assay (SPRIA) for WNV antibody was modified from that described by Rosenthal *et al.* (1973). WNV antigen was a freeze-thawed extract of WNV grown in Vero cells clarified by centrifugation. Control antigen was prepared in identical fashion from uninfected cells. Polyvinyl microtitre plates (Dynatech, Alexandria, Va., U.S.A.) were used to immobilize antigen. Rabbit $F(ab')_2$ anti-mouse $F(ab')_2$ (a gift from Dr A. F. Williams, MRC Cellular Immunology Unit, Oxford U.K.) was iodinated with ^{125}I-Na (carrier free) (Amersham International) by the method of Stagg *et al.* (1970) and used at 30000 to 50000 ct/min per well to detect any mouse anti-WNV antibody bound to WNV antigen. Results were expressed as a binding ratio (BR)

$$= \frac{\text{mean ct/min bound to WNV antigen}}{\text{mean ct/min bound to control antigen}}$$

The antibody titre was taken as the highest dilution of antibody giving a binding ratio of $\geqslant 2$. Positive and negative control sera were in each titration.

Antibody-dependent plaque enhancement (ADPE) assay. The 0·1 ml vol. of 10-fold (or 3·16-fold) dilutions of heat-treated (56 °C, 30 min) antiviral antiserum in complete MEM-α were distributed in 24 well multidishes (Flow Laboratories). Samples of 0·1 ml of a virus dilution chosen to give a countable number of plaques in control wells (no antibody) was added to each well and after 15 min incubation at 37 °C, 2×10^5 P388D1 cells in 0·3 ml vol. complete MEM-α were added. After a further 4 h incubation, 0·5 ml of overlay (complete MEM-α containing 1·5% CMC, final concn.) was added to each well to give a final concentration of CMC above cells of 0·75%. Plates were incubated for 3 to 5 days, according to virus type (see Table 1) until well-defined plaques appeared. The wells were washed in Dulbecco's phosphate-buffered saline (solution A) and stained with 0·1% naphthalene black for 30 min.

RESULTS

Antibody-dependent plaque enhancement assay

Fig. 1 shows WNV plaque enhancement produced by homologous rabbit antiserum. Two doses of virus, chosen to give countable plaques (approx. 50 and 5 p.f.u. per well) were used. At antibody dilutions of 10^{-2} to 10^{-4} there is marked enhancement of plaque counts (up to 40-fold). A 'prozone' in plaque enhancement is seen at high antibody concentrations (namely 10^{-1}) and is probably due to superimposed virus neutralization (the 50% PRNT titre versus WNV of the serum was 1/64).

Antisera to all flaviviruses tested enhance WNV plaque formation in P388D1 cells (Fig. 2). However, the 'prozone effect' is seen only in homologous virus antibody interactions or to a lesser degree when antibody to antigenically closely related viruses, e.g. Japanese encephalitis, is used. Antibody to antigenically unrelated viruses, e.g. Semliki Forest virus, has no effect in this system.

Effect of cell density

P388D1 cells do not show contact inhibition and it was found that if too high a cell density was used at the time of infection the cells overgrew and came off the plastic before virus plaques had time to form. With WNV (and other viruses that form plaques within 2 to 4 days, see Table 1), the optimal cell input per well was $1{\cdot}4 \times 10^5$ to 2×10^5. Slower growing viruses require cell inputs at the lower end of this range.

Reproducibility of the ADPE titration

The endpoints in serum ADPE antibody titrations were estimated by taking the highest dilution of antibody at which plaque counts are increased by threefold or more over controls (the factor of 3 being chosen empirically to give an easily reproducible endpoint).

Effect of preincubating virus–antibody mixtures before addition of cells

Fig. 3 (*a*) shows the kinetics of the ADPE assay, using different times of preincubation of WNV and homologous antibody. High concentrations of antibody neutralize WNV, although some enhancement is seen when 10^{-2} dilution of antibody is used without preincubation. At a 10^{-3} dilution, maximal enhancement is seen without preincubation, but at 10^{-4} and 10^{-5} dilutions of antibody, enhancement is slightly greater following preincubation than in its absence. When Japanese encephalitis virus antiserum is used with WNV, the pattern is similar to that seen with WNV antibody, but with a 'shift to the left', i.e. maximal enhancement occurs at 10^{-2} antibody dilution instead of 10^{-4} (Fig. 3 *b*). Japanese encephalitis virus is closely related to WNV whereas Louping-ill virus is a much more distantly related flavivirus.

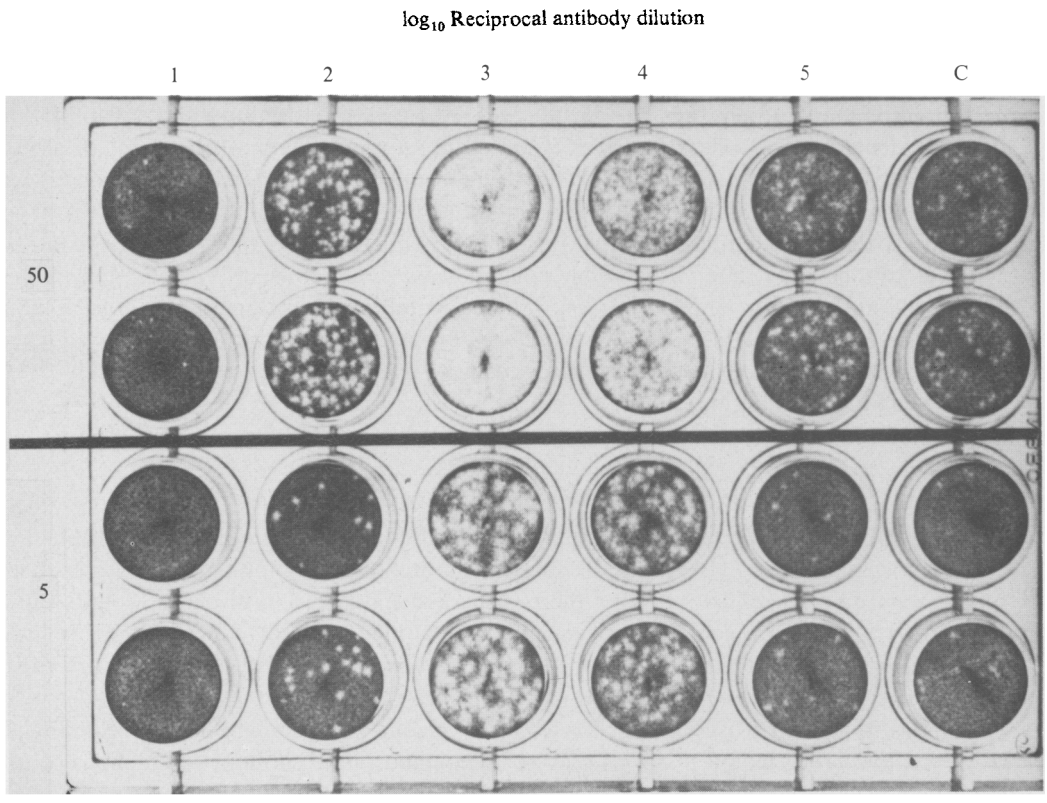

Fig. 1. Antibody-dependent plaque enhancement of WNV with rabbit anti-WNV immune serum. Tenfold dilutions of rabbit anti-WNV immune serum (PRNT titre 1/64) were mixed in duplicate with virus dilutions of approx. 5 and 50 p.f.u. per well and the titration completed as described in the text.

When dilutions of Louping-ill antiserum were mixed with WNV after different times of preincubation, the results shown in Fig. 3 (*c*) were obtained. There is a further shift to the left as compared to Japanese encephalitis antiserum.

Minimizing the time of preincubation gives plaque enhancement with the broadest range of antibody dilutions. It should be noted that the apparent ADPE titre increases with increasing duration of preincubation. In Fig. 3 (*a*), $\log_{10}$ reciprocal ADPE titres were 4·0 at 0 h and at 30 min, rising to 5·0 after 4 h preincubation.

Comparative sensitivity of the ADPE assay for antiviral antibody

Anti-WNV antibody titres as detected by 50% PRNT, HI, SPRIA and ADPE assays are compared on five anti-flavivirus-immune mouse ascitic fluids (Table 2). It is apparent that ADPE is as sensitive or more sensitive than SPRIA in detecting antiviral antibody and is a hundred times more sensitive than either HI or PRNT.

Viruses demonstrating ADPE in P388D1 cells

The viruses investigated are shown in Table 1. All the flaviviruses, alphaviruses and Bunyaviridae that show plaque formation on P388D1 cells also showed ADPE. The number

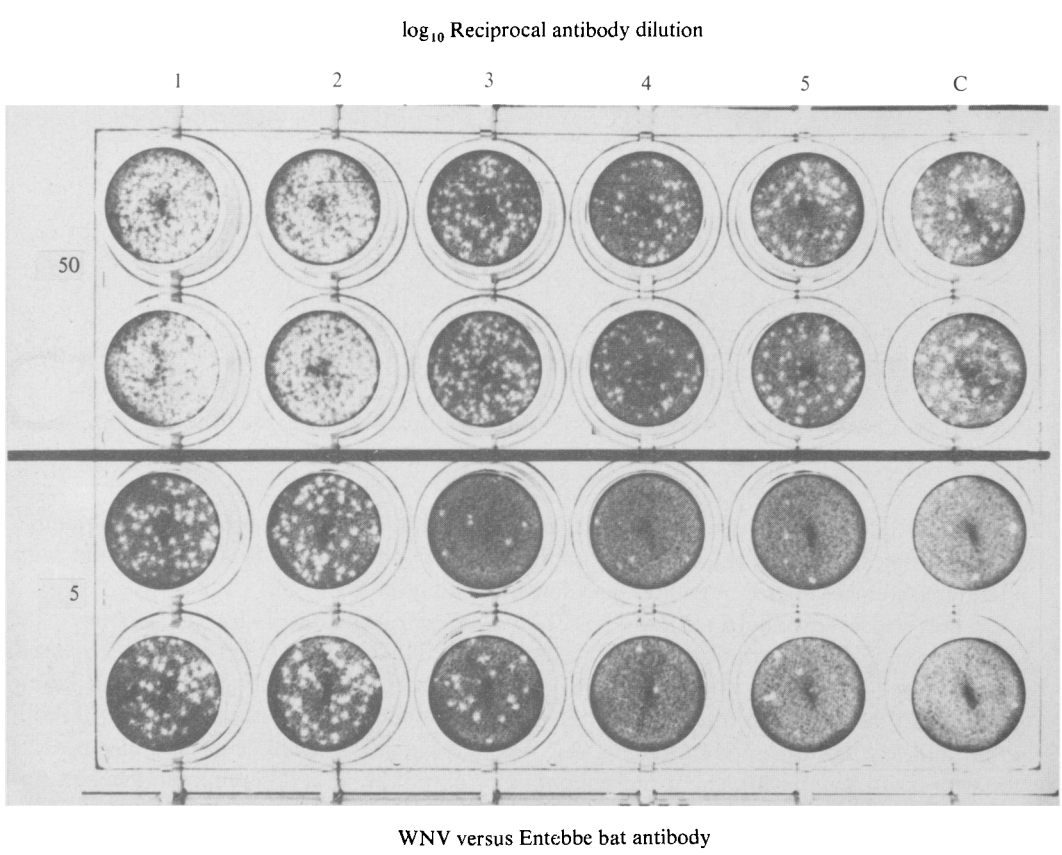

Fig. 2. Antibody-dependent plaque enhancement of WNV with rabbit anti-Entebbe bat virus immune serum. The procedure was as described in Fig. 1.

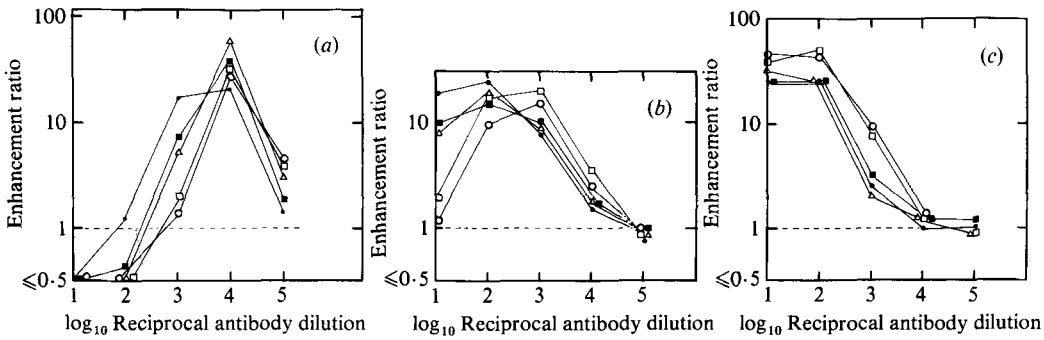

Fig. 3. Effect of preincubation of virus-antibody mixtures on the ADPE reaction. Tenfold dilutions of rabbit antisera against (*a*) WNV, (*b*) Japanese encephalitis virus and (*c*) Louping-ill virus were titrated by the ADPE assay using WNV, the P388D1 cells being added after the following periods of virus-antibody pre-incubation: ●, 0 h; ■, 0·5 h; △, 1 h; □, 2 h; ○, 4 h (– –, control). Enhancement ratio is equivalent to p.f.u. in the presence of antibody dilution divided by p.f.u. in the absence of antibody.

of days required for development of well-defined plaques is given in Table 1. On the other hand, although herpes simplex virus type 1 (Herpesviridae) and Mengo virus (Picornaviridae) formed plaques in P388D1 cells, ADPE was not demonstrated.

Table 2. *Comparative sensitivity of the antibody-dependent plaque enhancement assay*

	Reciprocal antibody titre to WNV			
Antibody*	50% PRNT†	HI†	SPRIA†	ADPE†
5628/C3	160	160	1250	10000
5628/A4	10	80	6250	10000
5635/A	10	80	1250	1000
5628/A1	40	160	6250	10000
5628/B1	40	40	1250	1000

* Mouse ascitic fluids prepared against WNV.

† PRNT, plaque reduction neutralization titre; HI, haemagglutination inhibition titre; SPRIA, solid-phase radioimmune assay; ADPE, antibody-dependent plaque enhancement assay, based on threefold enhancement titre.

DISCUSSION

ADPE in the P388D1 macrophage cell line is a highly sensitive, reproducible and simple means of detecting antiviral antibody, applicable to a number of virus families and with potential usefulness in epidemiology, serodiagnosis and research.

Of the viruses examined so far, ADPE was demonstrable with all the alphaviruses, flaviviruses and Bunyaviridae that form plaques on P388D1 cells. These groups of viruses, although being similar in sharing an arthropod-borne mode of transmission show considerable differences in their replicative cycles. The inability to demonstrate ADPE with herpes and Mengo viruses is intriguing. A possible explanation could lie in differences in the modes of virus attachment, entry and uncoating or alternatively in differences in mechanisms of neutralization ('single-hit' versus 'multi-hit' mechanisms).

The kinetics of the ADPE reaction seems to be similar to those observed by Hawkes (1964) in chick embryo fibroblasts (albeit plaque enhancement in the latter system was of much lower magnitude). The results are compatible with the concept that 'plaque enhancement is an intermediate stage along a reaction path leading to neutralization', an idea suggested by Hawkes (1964).

The presence of a prozone in ADPE at high antibody concentrations, especially in homologous virus-antibody systems, means that the use of single antibody dilutions in screening for antiviral antibody could potentially lead to some false negative results. The use of at least two antibody dilutions (e.g. 10^{-1} and 10^{-3}) would exclude such false negatives. Minimal preincubation of virus antibody mixtures ($\leqslant$30 min) would also help in reducing the range of antibody dilutions at which prozone effects occur.

Antibodies to flaviviruses closely related (e.g. Japanese encephalitis virus) or only distantly related (e.g. Louping-ill virus) to WNV show ADPE of comparable magnitude at optimal antibody dilutions although ADPE titres differ. Log_{10} reciprocal antibody titres were for WNV antiserum 4·0; for Japanese encephalitis virus antiserum 3·0; and for Louping-ill virus antiserum 3·0.

The experimental results reported here relate to antibodies produced in rabbits and mice, but other unpublished observations have shown that appropriate antisera of human or ovine origin will also enhance flavivirus replication in P388D1 cells, indicating that species homology between the macrophage and the immunoglobulin source is not essential. Some degree of complementarity at the animal class level may, however, be required between the Fc molecule and its receptor, since in chick embryo fibroblast cultures, which contain a subpopulation of cells of macrophage type, enhancement of viral plaque numbers is produced by antiviral antisera of avian, but not of mammalian origin. (Hawkes, 1964; Hawkes & Lafferty, 1967; Kliks & Halstead, 1980.)

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