Monoclonal antibodies identify the NS5 yellow fever virus non-structural protein in the nuclei of infected cells

A. Buckley,[1] Sofia Gaidamovich,[2] Anastasiya Turchinskaya[2] and E. A. Gould[1]*

[1]*NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, U.K. and*
[2]*WHO Collaborating Centre, D. I. Ivanovsky Institute of Virology, Moscow, Russia*

Eight monoclonal antibodies (MAbs) derived using yellow fever (YF) virus (French viscerotropic virus strain) labelled the nuclei (wild-type strains) and/or the nucleoli (vaccine strains) of cells infected with different strains of YF virus. The specificity of these antibodies for YF virus-infected cells was confirmed using MAbs that bind only the YF virus envelope glycoprotein. The characteristics of fluorescent labelling in the nuclei and nucleoli of both normal cells and of nuclei separate from cell cytoplasm confirmed that the antigen was inside the nucleus rather than on the outer surface of the nuclear membrane. Virus-specific antigen was also observed in the cytoplasm of infected cells. One additional virus envelope-specific antibody, derived at the same time, identified cytoplasmic antigen exclusively. The eight antibodies that identified nuclear antigen showed no activity in neutralization, haemagglutination inhibition or mouse protection tests. Analysis of their molecular specificities by radioimmunoprecipitation of virus-infected cell lysates showed that they identified the non-structural NS5 antigen of YF virus. These results support the possibility of nuclear involvement in the replicative stages of YF virus infection.

Introduction

Studies of the replication cycle of flaviviruses have generally shown that virus proteins occur only in the cytoplasm of infected cells. Unequivocal evidence that flavivirus-specific proteins can be found within the nuclei of flavivirus-infected cells has only recently been forthcoming. Its significance in virus replication and/or pathogenesis has not yet been determined. Previous reports of dense particles in the nuclei of cells infected with flaviviruses (Murphy, 1980; Tikhomirova *et al.*, 1968; Yasuzumi & Tsubo, 1965) had not been confirmed and evidence of a virus-specific step in the nucleus was unconvincing (Westaway, 1980). Recently, Buckley & Gould (1988) described two monoclonal antibodies (MAbs), prepared against yellow fever (YF) or West Nile (WN) virus, which produced fluorescent labelling of cytoplasmic antigen in cells infected with the virus used to derive the antibodies. However, when these antibodies were tested with Langat virus- (for the YF virus-derived antibody) or Zika virus-infected cells (for the WN virus-derived antibody), they produced virus-specific nuclear as well as cytoplasmic fluorescence. Independently, Tadano *et al.* (1989) also observed virus-specific nuclear fluorescence in dengue 4 virus-infected cells tested with core protein-specific MAbs derived from dengue 4 virus. We now present convincing support for involvement of the nucleus during the replication cycle of flaviviruses using MAbs prepared against the French viscerotropic virus strain of YF virus (YF FVV).

Methods

Viruses. A large number of flaviviruses and several different strains of YF virus were used. The sources and method of preparation of most viruses have been described previously (Gould *et al.*, 1985b; Buckley & Gould, 1985). The YF FVV and French neurotropic virus (YF FNV) were derived from YF virus isolated in Dakar, Senegal (1927) from a human (Mathis *et al.*, 1928). Their passage histories in monkeys, pig kidney-derived (PS) cells and mice are substantially different and were defined by Fitzgeorge & Bradish (1980). YF FVV was shown to have significantly reduced central nervous system (CNS) involvement in mice as compared with YF FNV, which was used as a YF vaccine in Africa and had good protective properties. YF FNV was eventually discontinued as a vaccine owing to occasional adverse reactions in younger vaccinees.

Cells. Vero cells were obtained from stocks held at the London School of Hygiene and Tropical Medicine and CV-1 cells were supplied by Dr J. C. S. Clegg, C.D.E., Porton Down, Salisbury, U.K. All cells were grown in Leibovitz L15 medium containing 5% foetal calf serum as described previously (Gould *et al.*, 1985b).

MAbs. The MAbs were produced against YF FVV by immunization of 8-week-old female BALB/c mice via the intraperitoneal route three times using infectious suckling mouse brain virus suspension (estimated 1×10^5 p.f.u. of virus per mouse). The spleens were removed and fused with SP2/0 cells using polyethylene glycol as described previously

(Gould *et al.*, 1985*b*). Two independent fusions were carried out using immune spleen cells from different mice. Both fusions yielded hybridomas that produced NS5-specific MAbs. Nine antibody-secreting hybridoma cell lines were selected by indirect immunofluorescence using YF 17D-infected acetone-fixed cells. The hybridomas were cloned by micromanipulation and injected into pristane-treated mice to produce high titre ascitic fluids.

Immunofluorescence. Cells in growth medium were infected in suspension with appropriate concentrations of virus and, after gentle agitation for 60 min, were seeded into plastic Petri dishes containing glass coverslips. They were incubated at 37 °C for the required incubation period. For indirect immunofluorescence microscopy, the biotin–streptavidin procedure was used to label the antigen in the infected cells (Gould *et al.*, 1985*a*). Unless otherwise stated, all cells were fixed with cold acetone prior to use. In all experiments the proportion of infected cells in the monolayer was monitored using a hyperimmune rabbit polyclonal antiserum against YF virus (Gould *et al.*, 1985*b*).

Nuclear monolayers. Virus-infected cells on glass coverslips were immersed in PBS containing 1·5% NP40 at 37 °C for 3 min, washed in PBS and fixed in acetone. When examined by phase-contrast microscopy the cytoplasmic contents of the remaining adherent cells had been removed leaving nuclei attached to the coverslips. The principle of this method has been reported previously (Bell, 1974).

Purification of nuclei. Cells were infected with virus as described above, seeded to confluence in 75 cm^2 tissue culture flasks and incubated for 48 h at 37 °C. The cells were collected in reticulocyte standard buffer (RSB; 0·01 M-Tris–HCl pH 7·7, 0·01 M-KCl, 0·015 M-$MgCl_2$). They were homogenized in a Dounce homogenizer and the nuclei sedimented at 500 g for 10 min. The nuclear pellet was washed twice in 0·25 M-sucrose in RSB containing 3 mM-$CaCl_2$. The pellet was resuspended in 7 ml of 0·25 M-sucrose in RSB and layered onto a gradient consisting of 2 M-sucrose (7 ml) with 3 mM-$CaCl_2$ and 7 ml of 1·6 M-sucrose in RSB. After centrifugation at 90000 g for 30 min, the nuclear band was collected, diluted in RSB and sedimented at 500 g for 10 min. The nuclei were resuspended in 1 ml of RSB, then dried onto slides and fixed using acetone (Shlomai & Becker, 1975).

Biological properties of the MAbs. The methods used for neutralization of infectivity, haemagglutination inhibition (HAI) and mouse protection studies have been described previously (Buckley & Gould, 1985; Gould *et al.*, 1985*b*).

Radioimmunoprecipitation (RIP). These procedures have been described in detail previously (Gould *et al.*, 1985*b*).

Double immunofluorescence labelling. MAbs that bound virus-specific nuclear antigen were incubated on infected cells on coverslips for 45 min, the coverslips were washed in PBS and anti-mouse biotinylated antibody was added for 45 min. The coverslips were washed and Texas Red conjugated with streptavidin (Amersham) was added for 10 min. The coverslips were washed again in PBS before addition of MAb 864 [specific for YF 17D virus envelope protein (see Gould *et al.*, 1985*b*)] conjugated with fluorescein isothiocyanate. The coverslips were washed and mounted in glycerol–saline pH 8·6.

Results

Immunofluorescence with MAbs

A panel of MAbs was produced in two separate experiments using SP2/0 myeloma cells fused with spleen cells from mice immunized with YF FVV. The nine MAbs described in Methods were titrated by indirect immunofluorescence (IIF) on acetone-fixed YF virus-infected Vero cells. All MAbs reacted strongly at dilutions up to 1:10000 and were subsequently used at 1:100 dilution. MAb JEL-2 produced distinct cytoplasmic fluorescence (Fig. 1*a*) typical of the pattern seen with MAbs specific for the envelope protein of YF virus. In contrast, the other eight MAbs (designated GJL-1, -5, -9, -10, -12, -14, -17 and -19) all produced pronounced fluorescent labelling in the nuclei of infected cells. Some cells were also labelled in the cytoplasm and along the plasma membranes (Fig. 1*b*). The above experiments were repeated using YF virus-infected BHK, PS, SW13, C6/36 and CV-1 cell lines with similar results. A polyclonal mouse antiserum against YF FVV was also tested by IIF using YF virus-infected Vero cells. Strong cytoplasmic fluorescence was seen (up to a dilution of 1:2000) in virus-infected cells but there was no obvious nuclear fluorescence.

Double fluorescence labelling

To demonstrate that the nuclear fluorescence identified virus-infected cells, a double labelling experiment was developed using fluorescein-labelled MAb 825 (Gould *et al.*, 1985*b*) specific for the cytoplasmic envelope protein of YF virus (Fig. 2*a*) and Texas Red-conjugated MAb GJL-5 that labelled nuclear antigen (Fig. 2*b*). By using this double labelling procedure, the cells with pronounced cytoplasmic fluorescence were found to have a strong nuclear label (Fig. 2*c*). In cells that were at the periphery of an infectious focus, i.e. cells at the early stages of the infectious cycle, only nuclear labelling was apparent.

Development of virus antigen during an infectious cycle

The YF 17D virus was inoculated onto monolayers of Vero cells at an estimated input multiplicity of 10. These infected monolayers were incubated at 37 °C and samples were tested by IIF microscopy for the presence of virus antigen at 4 h intervals. At about 12 h post-infection (p.i.) both nucleolar and nuclear fluorescence could be detected in the infected cells although the fluorescence was more pronounced in the nucleolus. Cytoplasmic fluorescence was not seen until 16 h p.i. This cytoplasmic fluorescence was most concentrated in the perinuclear region but could also be detected in the cytoplasm of some cells. By 20 h p.i. the nuclear fluorescence was very distinct and the cytoplasmic fluorescence had become more pronounced. By 30 h p.i., all cells contained large amounts of both nuclear and cytoplasmic fluorescent labelling. These results are
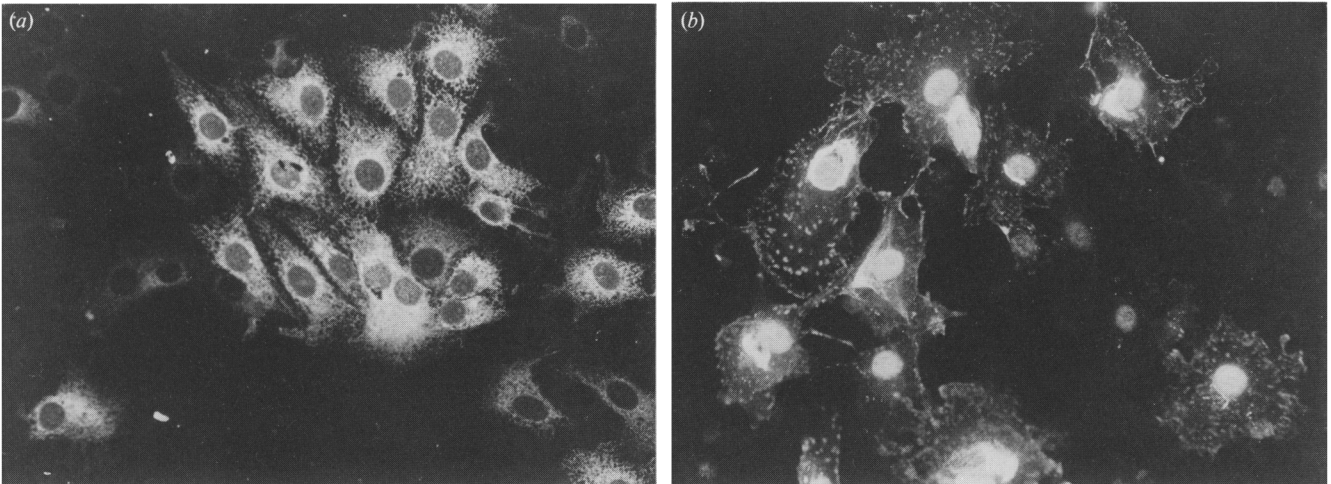
Fig. 1. IIF microscopy on acetone-fixed YF virus-infected cells (antibodies used at 1:1000 dilution). (*a*) Typical cytoplasmic fluorescence seen with E protein-specific MAbs. (*b*) Typical nuclear fluorescence seen with the NS5-specific MAbs which also labelled the cytoplasm and membrane of infected cells.
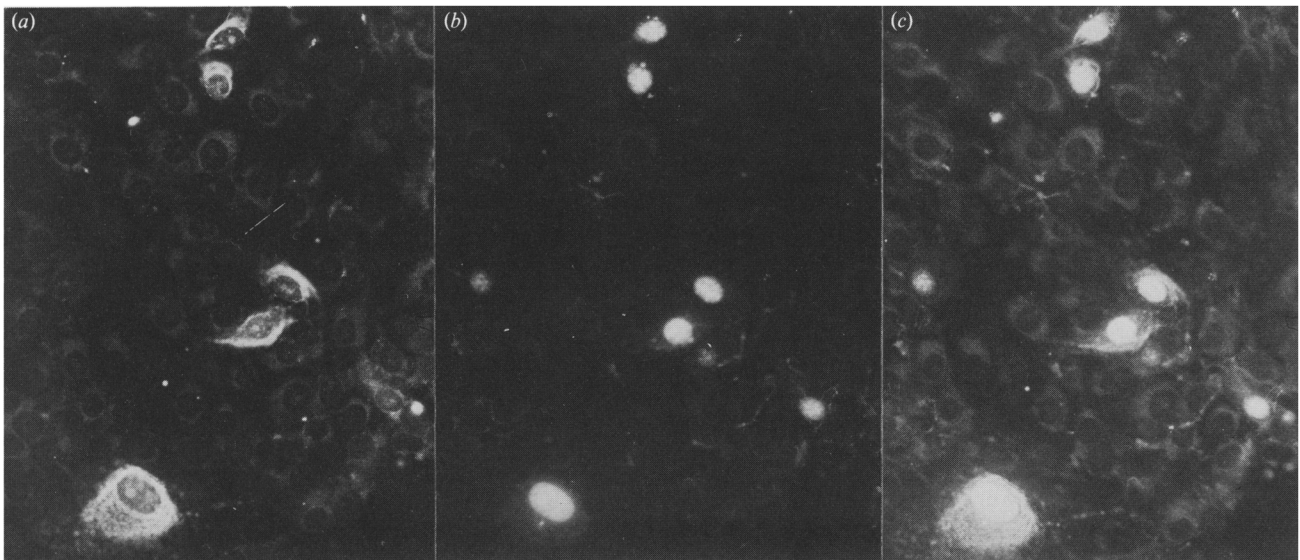

Fig. 2. Double fluorescence labelling of YF virus-infected cells. (*a*) MAb 825 labelling cytoplasmic antigen (fluorescent label) of infected cells. (*b*) The same cells labelled in the nuclei (Texas Red label) with MAb GJL-5, using a filter to exclude the cytoplasmic (fluorescent) label. (*c*) Both cytoplasmic and nuclear labelling can be seen in a doubly exposed photograph.

summarized in Table 1. These findings were similar for either vaccine or wild-type strains of YF virus.

Specificity of MAbs for YF virus

The MAbs were analysed by IIF microscopy using 40 different preparations of YF virus isolated from different regions of Africa, Central and South America and the Caribbean. These viruses included YF vaccine (17D,

17DD, FNV) and wild-type viruses (including YF FVV). All the MAbs reacted with all the viruses but two different and characteristic patterns of fluorescence were noted. All the vaccine viruses and also YF FVV produced fluorescence in the nucleoli of infected cells. This nucleolar fluorescence was evident in cells which were either at an early or a late stage of infection (Fig. 3*a*). In contrast, all wild-type YF viruses produced predominantly nuclear but not nucleolar fluorescence
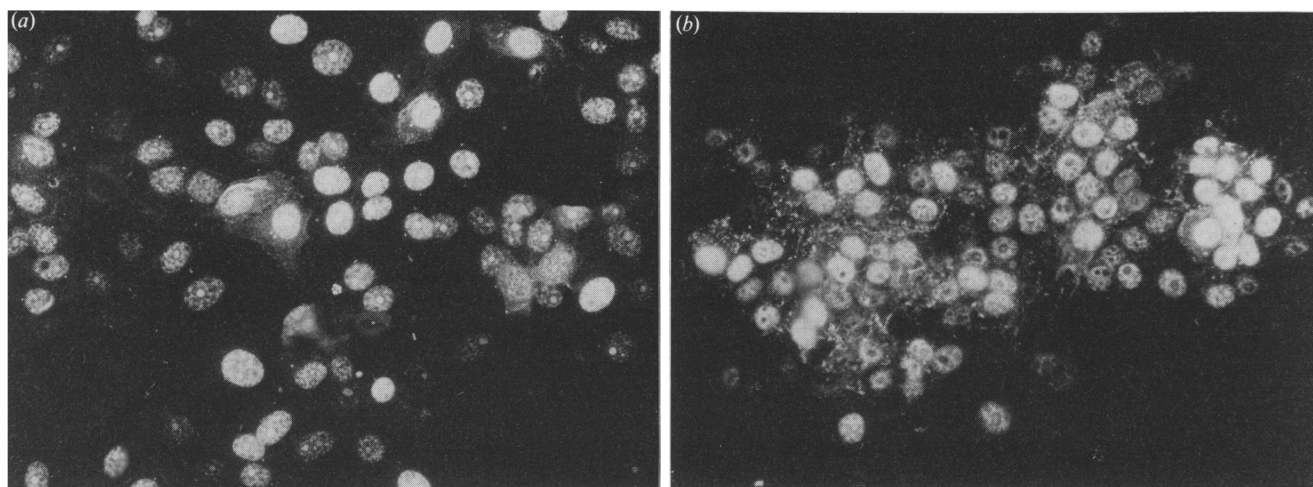
Fig. 3. (a) Nuclear fluorescence produced using NS5-specific MAb GJL-5 and YF 17D vaccine strain-infected cells. Note that in many cells both the nucleus and nucleolus are labelled. (b) Nuclear but not nucleolar fluorescence, as seen using wild-type YF viruses and MAb GJL-5.

Table 1. *Time course of production of YF 17D viral antigens in Vero cells detected with MAbs specific for the NS5 or envelope protein*

	MAbs against NS5 protein		MAb 825 (envelope protein-specific)
Time (h)	Cytoplasmic fluorescence*	Nuclear/nucleolar fluorescence	Cytoplasmic fluorescence
4	−	−	−
8	−	−	−
12	±	+	−
16	+	+ +	+
20	+	+ + +	+
24	+	+ + +	+ +
30	+ +	+ + +	+ + +

* Number of cells showing cytoplasmic fluorescence lower than those showing nuclear fluorescence. −, No cells showing fluorescence; ±, one or two cells showing very limited fluorescence; +, 1 to 5% of cells showing fluorescence; + +, 10 to 20% of cells showing fluorescence; + + +, 25 to 50% of cells showing fluorescence.

(Fig. 3b). The MAbs were also compared by IIF for their ability to bind to 56 other viruses classified in the genus Flavivirus. MAb JEL-2, which labelled only cytoplasmic antigen, showed cross-reactivity with Japanese encephalitis, Spondweni and Uganda S viruses. The remaining MAbs, i.e. the eight that identified nuclear antigen, failed to react with any flaviviruses other than YF virus.

MAb biological activity

Each MAb that labelled nuclei was analysed in functional tests for HAI, neutralization of virus infectivity and mouse protection against virulent YF virus

challenge. None of these MAbs showed biological activity in any of the tests performed. MAb 208 inhibited YF FVV haemagglutination and also neutralized the infectivity of this virus in plaque reduction neutralization tests.

Specificity of the MAbs for the nuclei of infected cells

In addition to the evidence presented above, two further methods were used to demonstrate that the fluorescent antigen was present in the nuclei of the infected cells. In the first method, NP40 was used to separate cytoplasmic membranes from nuclei. A variety of conditions was employed to obtain the most suitable treatment to remove cytoplasmic membranes. On the basis of phase contrast microscopy and immunofluorescence using MAb GJL-5 (Fig. 4a), a concentration of 1·5% NP40 with an immersion time of 3 min was the most satisfactory. The appearance of the same infected cells, when analysed by IFF microscopy with nuclear labelling MAb GJL-5, is shown in Fig. 4(b). Nuclei purified from YF virus-infected Vero cells, using Dounce homogenization (see above), were dried and fixed onto coverslips for IIF microscopy. The results confirmed those obtained with the nuclear monolayers. In both types of analysis, MAb JEL-2, which specifically identifies cytoplasmic antigen, was negative.

Examination of the molecular specificity of the MAbs

The molecular specificity of the MAbs was determined using each MAb in RIP tests with [^{35}S]methionine-labelled YF virus-infected CV-1 cells. The immunoprecipitated viral proteins were analysed by PAGE on 12%
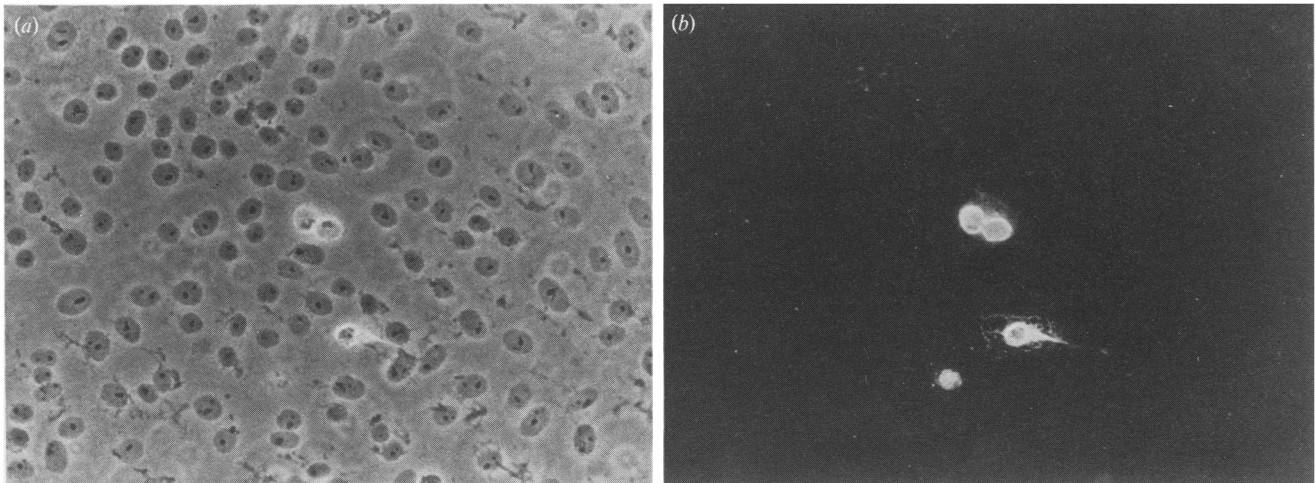
Fig. 4. (a) Phase contrast microscopy showing nuclear monolayers in which most of the cytoplasm has been removed leaving naked nuclei. (b) The same preparation showing nuclear fluorescence (MAb GJL-5) in four nuclei.
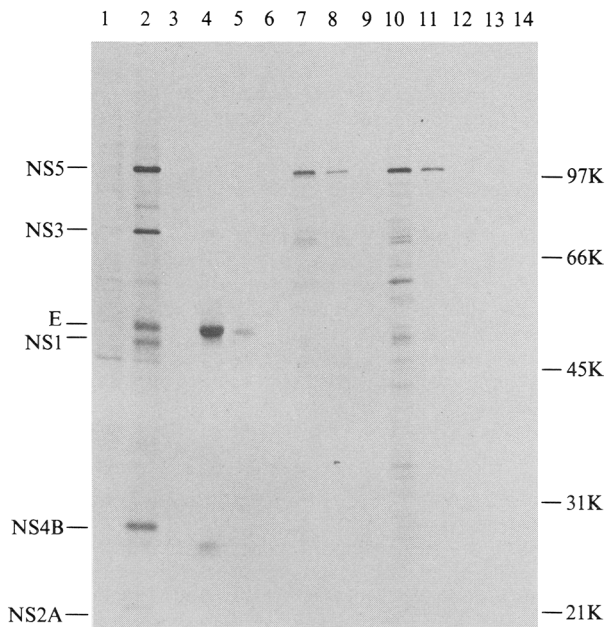

Fig. 5. RIP analysis with [^{35}S]methionine-labelled YF virus-infected CV-1 cell lysates. Lanes 1, 3, 6, 9 and 12, mock-infected; lanes 2, 4, 7, 10 and 13, YF 17D virus-infected; lanes 5, 8, 11 and 14, YF Asibi virus-infected. The first two lanes are a direct PAGE analysis. The remaining lanes are RIP tests with appropriate MAbs. MAb JEL-2, E protein-specific (lanes 3, 4 and 5); MAb GJL-5, NS5-specific (lanes 6, 7 and 8); MAb GJL-1, NS5-specific (lanes 9, 10 and 11). Lanes 12 to 14 contain control antibody for mock infection, YF 17D virus infection and YF Asibi virus infection.

polyacrylamide gels. The results are presented in Fig. 5. MAb JEL-2 produced a distinct band with an M_r of 54K which corresponds with the known size of YF virus envelope glycoprotein. The eight MAbs that labelled nuclei all precipitated a protein of 102K, an M_r which corresponds to that of the NS5 protein of YF virus. A weak protein band of 76K was also seen in some gels and probably represents coprecipitated NS3 protein.

Discussion

Eight MAbs against YF FVV were specific for YF virus, failing to bind any of 56 other flaviviruses. None of these MAbs showed any biological activity in HAI, neutralization or mouse protection tests. The most remarkable aspect of these MAbs was their ability to identify a YF virus antigen in the nuclei and in some cases also in the nucleoli of infected cells. As can be seen in Fig. 1 to 4, the appearance of the nuclear antigen was striking, particularly in those examples which showed strongly labelled nuclei but non-labelled nucleoli. The precision with which nuclear and nucleolar labelling could be independently defined, together with the data obtained using double fluorescence labelling, nuclear monolayers and purified nuclei, supports the conclusion that at least some of the fluorescent labelling was inside the nucleus rather than on the outside surface of the nuclear membrane. It is not clear why vaccine and wild-type YF virus were so distinctively different in their capacity to produce a detectable antigen in the nucleolus of the infected cells. However, this could result from the selective effects of long-term culturing of vaccine strains in tissue culture cells or animals. It is also difficult to understand why so many (i.e. eight of nine) MAbs that were specific for the NS5 protein were obtained in two fusions, since MAbs with specificity for NS5 have not been reported previously. The fact that a polyclonal

antiserum against YF FVV did not show distinct nuclear fluorescence probably reflects the masking effect of cytoplasmic antigens and also the relatively lower titres of antibody reactivity usually observed with polyclonal antisera. Whether or not YF FVV has a strongly immunogenic NS5 protein has not yet been determined. A possible explanation for the predominance of NS5 MAbs derives from the observation that YF FVV is viscerotropic and can elicit protective immune responses in young mice (Fitzgeorge & Bradish, 1980). In contrast YF FNV, which replicates in mouse brain cells, and the YF 17D vaccine strain of YF virus do not induce protective immune responses. Thus, the viscerotropic nature of YF FVV possibly favours the induction of antibody responses against a wide range of virus-encoded proteins.

The specificity of these MAbs for the YF virus NS5 protein is pertinent since the NS5 protein is the putative viral polymerase (Westaway et al., 1987). Moreover, Rice et al. (1986) showed that residues 3037 to 3181 of YF virus NS5 are conserved amongst the RNA-dependent polymerases of 10 positive-stranded RNA viruses. Recent evidence suggests that during viral replication, the flavivirus RNA forms a replication complex of dsRNA, NS5 and possibly NS3, associated with nuclear membranes (V. Shamanin, personal communication). Our observation that NS3 coprecipitated with the NS5 protein supports these ideas. Although early evidence of flavivirus-specific antigen in infected cell nuclei (Murphy, 1980; Tikhomirova et al., 1968; Yasuzumi & Tsubo, 1965) was considered controversial (Westaway, 1980), our evidence presented with YF virus NS5 MAbs seems very convincing and this probably reflects the use of high titre MAbs together with the fortuitous availability of these MAbs. In addition, both we and others have previously reported the identification of other flavivirus antigens, i.e. E, NS1 and C, in the nuclei of infected cells (Buckley & Gould, 1988; Tadano et al., 1989). Whether or not the appearance of these nuclear antigens represents the identification of flavivirus replicative processes in nuclei has not yet been determined but further studies of these processes are taking place.

References

BELL, D. (1974). DNA synthesis in nuclear monolayers from BSC-1 cells infected with herpes virus. *Nature, London* **248**, 505–508.

BUCKLEY, A. & GOULD, E. A. (1985). Neutralization of yellow fever virus studied using monoclonal and polyclonal antibodies. *Journal of General Virology* **66**, 2523–2531.

BUCKLEY, A. & GOULD, E. A. (1988). Detection of virus-specific antigen in the nuclei or nucleoli of cells infected with Zika or Langat virus. *Journal of General Virology* **69**, 1913–1920.

FITZGEORGE, R. & BRADISH, C. J. (1980). The *in vivo* differentiation of strains of yellow fever virus in mice. *Journal of General Virology* **46**, 1–13.

GOULD, E. A., BUCKLEY, A. & CAMMACK, N. (1985*a*). Use of the biotin-streptavidin interaction to improve flavivirus detection by immunofluorescence and ELISA tests. *Journal of Virological Methods* **11**, 41–48.

GOULD, E. A., BUCKLEY, A., CAMMACK, N., BARRETT, A. D. T., CLEGG, J. C. S., ISHAK, R. & VARMA, M. G. R. (1985*b*). Examination of the immunological relationships between flaviviruses using yellow fever virus monoclonal antibodies. *Journal of General Virology* **66**, 1369–1382.

MATHIS, E., SELLARDS, A. J. & LAIGRET, J. (1928). Sensibilité du macacus rheses au virus de la fièvre jaune. *Compte rendu de l'Académie des sciences* **186**, 604–606.

MURPHY, F. A. (1980). Morphology and morphogenesis. In *St. Louis Encephalitis*, pp. 65–103. Edited by T. P. Monath. Washington, DC: American Public Health Association.

RICE, C. M., STRAUSS, E. G. & STRAUSS, J. H. (1986). Structure of the flavivirus genome. In *Togaviridae and Flaviviridae*, pp. 279–326. Edited by S. Schlesinger & M. J. Schlesinger. New York: Plenum Press.

SHLOMAI, J. & BECKER, Y. (1975). Analysis of herpes simplex virus DNA synthesized *in vitro* in nuclei from infected BSC-1 cells. *Virology* **68**, 275–279.

TADANO, M., MAKINO, Y., FUKUNAGA, T., OKUNO, Y. & FUKAI, K. (1989). Detection of dengue 4 virus core protein in the nucleus. I. A monoclonal antibody to dengue 4 virus reacts with the antigen in the nucleus and cytoplasm. *Journal of General Virology* **70**, 1409–1415.

TIKHOMIROVA, T. I., KARPOVICH, L. G., REINGOLD, V. N., LEVKOVICH, E. N. & SHESTOPALOVA, N. M. (1968). Comparative study of Langat and tick-borne encephalitis viruses in cultured cells. *Acta virologica* **12**, 529–534.

WESTAWAY, E. G. (1980). Replication of flaviviruses. In *The Togaviruses*, pp. 531–577. Edited by R. W. Schlesinger. New York: Academic Press.

WESTAWAY, E. G. (1987). Flavivirus replication strategy. *Advances in Virus Research* **33**, 45–90.

YASUZUMI, G. & TSUBO, I. (1965). Analysis of the development of Japanese B encephalitis (JBE) virus. III. Electron microscope studies on inclusion bodies appearing in neurons and microglial cells infected with JBE virus. *Journal of Ultrastructure Research* **12**, 317–327.