**Detection of Virus-specific Antigen in the Nuclei or Nucleoli of Cells Infected with Zika or Langat Virus**

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**SUMMARY**

Two monoclonal antibodies (MAbs) with molecular specificities for either the viral envelope glycoprotein (MAb 541) or the non-structural NS1 glycoprotein (MAb 109) were derived using West Nile and yellow fever (YF) viruses respectively. Their antigenic reactivity with a large number of flaviviruses was tested by indirect immunofluorescence microscopy. Both produced cytoplasmic fluorescent staining patterns with the homologous virus against which they were raised. Additionally, MAb 541 reacted with two substrains of YF virus whereas MAb 109 reacted with Bussuquara, YF and Ntaya viruses. These reactions were exclusively cytoplasmic. Two unexpected patterns of fluorescent labelling were observed when the antibodies were tested with Zika and Langat viruses. MAb 541 produced fluorescent staining of the nuclei, but not the cytoplasm, of cells infected with Zika virus and MAb 109 labelled only the nucleoli of cells infected with Langat virus. Double-labelling experiments showed that the nuclear fluorescent label was confined to virus-infected cells, and antibody absorption experiments with virus-infected cell packs confirmed the virus specificity of the nuclear antigen. The unexpected presence of virus-specific antigen in the nuclei or nucleoli of Zika or Langat virus-infected cells brings into question the role of the nucleus in flavivirus replication.

**INTRODUCTION**

The replication cycle of viruses in the family Flaviviridae (Westaway et al., 1985) is thought to be exclusively cytoplasmic (reviewed by Westaway, 1980). Morphogenetic studies reveal apparently mature virus particles associated with internal cell membranes (reviewed by Murphy, 1980) and also in the extracellular spaces of infected cells (Gould et al., 1985a). No definite budding mechanism has been described, and it is not entirely clear how the virions are released from the cell, although a process of exvagination is believed to be the most likely mechanism (reviewed by Westaway, 1980). Using immunofluorescence microscopy and appropriate antibodies that inhibit haemagglutination, virus-specific haemagglutinin has been identified in the cytoplasm either associated with the external side of the nuclear membrane, the Golgi apparatus, dispersed as inclusions throughout the cytoplasm, on the surface of infected cells or on the mature virions released from the cell (Cardiff et al., 1973; Cardiff & Lund, 1976; Gould et al., 1985a). A 48K non-structural glycoprotein, present in yellow fever (YF) virus-infected cells, has also been described in the perinuclear region of the cytoplasm or as a protein dispersed throughout the cytoplasm (Gould et al., 1985a). Previous reports of dense particles in the nuclei of cells infected with flaviviruses (Yasuzumi & Tsubo, 1965; Tikhomirova et al., 1968) have not been confirmed and evidence of a virus-specific step in the nucleus has now been ruled out (Westaway, 1980). Thus there is no convincing evidence that virus-specific proteins are present within the nuclei of flavivirus-infected cells.
Using monoclonal antibodies (MAbs) in indirect immunofluorescence tests, we report for the first time the presence of flavivirus-specific antigens in the nuclei of vertebrate and invertebrate cells infected with either Zika or Langat (LGT) virus.

METHODS

**Viruses.** A large number of flaviviruses and several different strains of YF virus were used. The sources and method of preparation of most were described previously (Gould et al., 1985a). Additional flaviviruses (see Table 1) were very kindly supplied by Dr J. S. Porterfield (Sir William Dunn School of Pathology, University of Oxford, U.K.) and Dr R. E. Shope (Yale Arbovirus Research Unit, New Haven, Conn., U.S.A.).

**Cells.** Vero cells were obtained from three separate sources, C1008 from Dr J. Casals (Yale Arbovirus Research Unit), W. Vero from Dr C. Brand (Wellcome Biotechnology, Beckenham, U.K.) and Vero from our own stocks that have been held in the London School of Hygiene and Tropical Medicine for at least 10 years. The invertebrate cell line AP61 has been described by Varma et al. (1973). BHK-21 cells were obtained from Flow Laboratories.

**Media.** All cells were grown in Leibovitz L15 medium containing 10% foetal calf serum (FCS) as described previously (Gould et al., 1985a).

**Plaque purification.** Vero cells infected with Zika virus were overlaid with Eagle's minimal essential medium containing 2% FCS and 0.5% Seakem agarose (FMC, Rockland, Me., U.S.A.). After incubation for 7 days at 37 °C, well separated plaques were removed with a Pasteur pipette and suspensions (1 ml/plaque) in medium were inoculated intracerebrally into newborn mice. Twenty percent suspensions of infected mouse brain were prepared when the infected mice were moribund.

**Preparation of MAbs.** Ten-week-old female BALB/c mice were inoculated intraperitoneally with a 1/100 dilution in phosphate-buffered saline (PBS) of a 20% suckling mouse brain suspension of either West Nile (WN) or YF Asibi (YF AS) virus. Eight days later spleens were removed and the cells were fused with NSO myeloma cells as described previously (Gould et al., 1985a). The supernatant medium from cloned cultures was tested for antibody production using the appropriate virus-infected C1008 cells by indirect immunofluorescence tests. All positive cultures were inoculated into pristane-treated adult male BALB/c mice to produce high titre MAbs. These cell cultures were cloned by micromanipulation and clones were stored under liquid nitrogen. MAB 813, which reacts in immunofluorescence tests with a very large number of flaviviruses, was prepared and characterized as described previously (Gould et al., 1985a).

**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 either 37 °C (vertebrate cells) or 28 °C (AP61 cells) for the required incubation period. For indirect immunofluorescence the biotin-streptavidin procedure was used (Gould et al., 1985b). All cells were fixed with cold acetone prior to use. In all experiments the proportion of infected cells for each virus preparation was monitored with a rabbit hyperimmune antiserum, RH2 or RH1 against WN or YF virus respectively (Gould et al., 1985a).

**Gel diffusion.** The isotype of each MAb was shown to be IgG2a by Ouchterlony gel diffusion using subclass-specific mouse anti-globulins (Miles Laboratories).

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

**Immunoblotting.** Infected cell lysate (2 x 10⁶ cells) was added to 0.5 ml of lysis buffer (10% glycerol, 1% SDS, 50 mM-Tris–HCl pH 7.5, 2 mM-PMSF, 1 mM-EDTA) and boiled for 2 min. The samples were electrophoresed on 10% SDS–polyacrylamide gels, electroblotted onto nitrocellulose paper which was then blocked by incubation in 5% dried milk powder in PBS with 0.1% Tween 20 for 30 min at room temperature. Blots were incubated with antibody (1:200 dilution) overnight at 4 °C. After washing in PBS with 0.1% Tween 20, the blots were incubated with affinity-purified horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-Rad) for 1.5 h at room temperature. They were then washed in PBS with 0.1% Tween 20, then PBS alone, and developed using diaminobenzidine in PBS with hydrogen peroxide.

**Antibody absorption experiments.** Confluent monolayers of C1008 cells were infected with Zika, WN, LGT or YF viruses and incubated at 37 °C until heavily infected but without serious destruction of the cells. These infected monolayers were washed with PBS, scraped from the glass and centrifuged at 1500 g for 15 min. The cell packs were fixed in cold acetone, washed thoroughly in PBS and frozen at -70 °C. Control non-infected cell packs were prepared in the same manner. The MAbs were diluted 1/100 in PBS and mixed with the appropriate cell packs. They were then incubated at 37 °C for 1 h. The absorption mixtures were clarified by centrifugation at 10000 g for 30 min and the procedure was repeated. MAbs treated in this way were then titrated by indirect immunofluorescence using the relevant virus-infected cells.

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

Antigenic characterization of MAbs by indirect immunofluorescence

MAbs 541 and 109 were used in indirect immunofluorescence tests on Vero cells infected with 42 flaviviruses representing each of the seven recognized antigenic subgroups and with 35 vaccine and wild-type strains of YF virus. MAb 541 reacted only with cells infected with either WN, YF-FNV (French neurotropic vaccine strain), YF TR4205 (a wild-type isolate) or Zika virus. The characteristic pattern of cytoplasmic fluorescence produced on WN-infected Vero cells is shown in Fig. 1 a. This cytoplasmic fluorescence was also seen with cells infected by YF virus but not Zika virus. It was surprising that YF-FNV and YF TR4205 were positive since no other strains of YF virus reacted in this test.

In contrast, virus immunofluorescence was observed as described below using MAb 541 and Zika virus-infected Vero cells (Fig. 1 b). In some cells, fluorescence was present both in the nucleolus and in other parts of the nucleus. In other cells, the nucleoli were not fluorescent even though there was nuclear fluorescence. There was no cytoplasmic fluorescence using acetone-fixed infected cells. However, when Zika-infected cells were fixed with cold methanol for 5 min, cytoplasmic but not nuclear fluorescence was observed. On the other hand it proved possible to produce both cytoplasmic and nuclear fluorescence with Zika-infected cells if they were first fixed with methanol, treated with MAb 541 and then fixed with acetone followed by further treatment with MAb 541. From these results we conclude that the cytoplasmic antigen was sensitive to acetone and that methanol fixation did not cause the nuclear membrane to become permeable to antibody; hence, lack of nuclear fluorescence with methanol-fixed cells resulted from the failure of MAb 541 to penetrate the nuclear membrane.

Estimates of the proportions of cells with nuclear fluorescence always corresponded closely with the estimated proportions of Zika-infected cells. At no time was nuclear fluorescence observed with non-infected cells when MAb 541 was used.

MAb 109 reacted only with cells infected with YF (all strains), Bussuquara, Ntaya or LGT virus. The cytoplasmic fluorescence produced by MAb 109 on YF-infected cells was perinuclear in distribution (Fig. 1 c). However, LGT virus infection was unusual in that, instead of showing cytoplasmic fluorescence, the fluorescent label was confined to the nucleoli of a proportion of the infected cells. This is demonstrated in Fig. 1 (d) which is a combined phase-contrast and immunofluorescence photograph. No cytoplasmic fluorescence was ever observed with LGT-infected cells if they were tested with MAb 109 alone. Immunofluorescence was never seen in non-infected cells.

Virus specificity of nuclear fluorescence

A series of experiments was performed to see whether or not both types of nuclear fluorescence were virus-specific. First, both hybridoma cell lines (541 and 109) were recloned by micromanipulation and five separate cloned cell lines of each were obtained. These were tested as above and the results were identical to those reported. Second, in separate experiments, Vero cells were infected with either Zika or LGT virus which had been plaque-purified. Each was then tested by indirect immunofluorescence with MAb 541 or 109 as already described. Again, the results were identical to those reported above, reducing the likelihood of a contaminating agent being responsible for the nuclear fluorescence.

Vero cells were infected with plaque-purified Zika or LGT virus at a range of input multiplicities from 0.1 to 0.0001. The infected cells were incubated at 37 °C for 24, 48, 72, 96 or 120 h. At each time interval, sample cultures were tested by double-labelling for the presence of either nuclear or cytoplasmic fluorescence using MAb 541 (nuclear) and MAb 813 (cytoplasmic) or MAb 109 (nucleolar) and MAb 813 (cytoplasmic) respectively for the appropriate viruses. With Zika virus, the proportion of double-labelled cells, i.e. with both nuclear and cytoplasmic fluorescence (Fig. 1 e), always corresponded to virus input multiplicity and the proportion of doubly fluorescent cells increased daily. This confirmed the observation that only cells infected with Zika virus produced nuclear fluorescence. With LGT virus, the proportion of cells with cytoplasmic fluorescence was always higher than the proportion showing nucleolar fluores-
Fig. 1. Indirect immunofluorescence using Vero cells. (a) MAb 541 and WN-infected cells; (b) MAb 541 and Zika-infected cells; (c) MAb 109 and YF-infected cells; (d) MAb 109 and LGT-infected cells; (e) MAb 541 and MAb 813 and Zika-infected cells; (f) MAb 541 and Zika-infected nuclear monolayer; (g) phase-contrast of MAb 541 and Zika-infected nuclear monolayer. Bar marker represents 10 μm.
Zika virus nuclear fluorescence

Vero cells, C1008, W. Vero, BHK-21 and the invertebrate cell line AP61 were each infected with Zika virus for immunofluorescence tests with MAb 541. These cell lines were also separately infected with LGT virus for tests with MAb 109. With Zika virus, nuclear fluorescence was observed in both vertebrate and invertebrate cell lines when tested with MAb 541. The nuclear fluorescence was particularly pronounced in virus-infected AP61 cells and, since they did not produce a cytopathic effect under the experimental conditions employed, we induced a persistent infection by subculturing the infected AP61 cells. These were then tested with MAb 541. Within 6 days of the initial infection with Zika virus, all nuclear fluorescence had disappeared despite the continued presence of Zika-specific cytoplasmic fluorescence (demonstrated using MAb 813). Nuclear fluorescence did not reappear in these infected cells when subcultured to undergo a persistent infection.

The results obtained with LGT virus and MAb 109 in AP61 cells were as reported with mammalian cells. We also induced a persistent infection of LGT virus in C1008 cells by subculturing the infected cells. This persistently infected cell line was examined at regular intervals over a period of months by indirect immunofluorescence with MAbs 109 and 813. Nuclear fluorescence was visualized in a low proportion of infected cells for the first 48 h after each subculture. Subsequently, the fluorescence disappeared until the next subculture was prepared. Thus, the appearance of virus-specific antigen occurs in the nucleus of several mammalian cell types and an invertebrate cell line under a variety of experimental conditions.

Nuclear monolayers of Zika-infected Vero cells were prepared and tested by immunofluorescence with MAb 541 as described in Methods. Virus-specific antigen was seen in the nuclei (Fig. 1f). A phase-contrast picture, without immunofluorescence, of the same cells is shown in Fig. 1(g); small dark inclusions are apparent and these coincide with the fluorescent particles seen in Fig. 1(f).

MAb 541, with a titre by immunofluorescence of 1/5000, was diluted 1/100 and then mixed with acetone-fixed cell packs containing either WN- or Zika-infected cells as described in Methods. For controls, non-infected Vero cell packs were used. The clarified supernatant-absorbed antibody preparations were tested by indirect immunofluorescence using WN- and Zika-infected Vero cells. Absorption of MAb 541 by either WN or Zika cell packs completely removed its ability to produce either cytoplasmic or nuclear fluorescence. The same procedure was also carried out using MAb 109 and YF AS- or LGT-infected cell packs. Absorption of the antibody completely removed its ability to produce either cytoplasmic or nuclear fluorescence.

Specificity of MAbs 541 and 109

Radioimmunoprecipitation (RIP) tests were performed with MAb 541 using 35S-labelled WN virus-infected cell lysates. MAb 541 immunoprecipitated only the envelope protein (Mr 53K) of WN virus (Fig. 2). Attempts to perform similar tests with Zika virus-infected cell lysates resulted in very poor labelling of the envelope protein. However, immunoblotting (see Methods) proved more satisfactory, and the specificity of MAb 541 for the envelope protein of Zika virus (two bands at Mr 54K and 56K) and control WN virus (one band at 52K) was confirmed (Fig. 3). Further controls for this test included a glycoprotein E-specific MAb (MAb 612) described previously (Gould et al., 1985a). In separate experiments, MAb 109 was used in RIP tests with YF-infected Vero cell lysates. This antibody precipitated the non-structural NS1 (46K to 48K) glycoprotein of YF virus (Fig. 4). Controls for this test included E-specific MAb 864, which precipitates E protein (53K) together with presumed cleavage products (46K to 49K), and MAb 979 which is NS1-specific (Gould et al., 1985a). All attempts to identify the molecular specificity of MAb 109 for LGT virus proteins have failed. This is presumed to be due to the very small quantities of nuclear antigen identified in the infected cells. The results obtained by molecular analysis essentially confirm those predicted from the biological data.
DISCUSSION

We have previously identified a MAb with specificity for a Japanese encephalitis virus non-structural antigen that also identified a nuclear antigen in both vertebrate and invertebrate cells (Gould et al., 1983). The nuclear antigen however was of cellular origin and was not encoded by the viral genome. As far as we are aware, a virus-specific nuclear antigen has never previously been described with flavivirus-infected cells, although dense intranuclear particles, no longer considered to represent virus-specific material (Westaway, 1980), were described in St Louis, Japanese and tick-borne encephalitis virus-infected cells (Murphy et al., 1980; Yasuzumi & Tsubo, 1965; Tikhomirova et al., 1968). However, the nucleus has been reported to be involved in flavivirus biosynthetic events (Pfefferkorn & Shapiro, 1974). It was surprising that a MAb induced against WN virus, and possessing only a very limited cross-reactivity with other flaviviruses, produced nuclear fluorescence in Zika virus-infected cells and moreover that a totally unrelated MAb prepared against YF AS virus produced nucleolar fluorescence in cells infected with LGT virus. Whether these results indicate differences in post-translational processing of polyproteins with different flaviviruses needs to be investigated. It is possible that different flaviviruses accumulate different relative amounts of antigen in the nucleus. Alternatively, if there is a nuclear step for WN and YF virus replication which has not yet been identified, the epitopes represented by MAbs 541 and 109 may not be present in the relevant antigens. It must be assumed that flavivirus antigens are not frequently observed in the nuclei of infected cells because most antibody preparations do not contain the relevant specificities. In addition, the use of polyclonal antisera might fail to reveal nuclear antigens due to masking of the nuclear fluorescence by cytoplasmic fluorescence.
In view of the controversial nature of our observations we performed a series of experiments to confirm that the fluorescence was in the nucleus and did arise as the result of infection of the cells with Zika and LGT viruses. Several of the experiments showed that only cells with virus antigen in the cytoplasm produced nuclear fluorescence. By recloning the MAb we confirmed it was not contaminated with mouse antibody against adventitious antigens. Furthermore, the choice of both vertebrate and invertebrate cells ruled out the possibility that we had merely detected a contaminating antigen in the Vero cell line. This was also confirmed by plaque purification of the virus. Double-fluorescence labelling experiments demonstrated that the nuclear antigen was induced only in cells infected with virus and the nuclear monolayers provided evidence that at least some of the antigen was inside the nucleus. In view of the fact that we used MAbs, it seems likely that the antigen on the outside of, or in, the nuclear membrane is the same as that inside the nucleus and further evidence for this comes from the antibody absorption experiments. However, despite these controls, we are unable to exclude totally the unlikely possibility of the presence of a contaminating virus in the Zika and LGT inocula which is antigenically related to flaviviruses.

Analysis showed that MAb 541 recognized an epitope of the flavivirus envelope protein and MAb 109 recognized flavivirus non-structural NS1 glycoprotein. While it seems unlikely that envelope protein enters nuclei of infected cells, it may be present on the outside of the nucleus; antigen inside the nucleus could be a part of a precursor polyprotein synthesized before virus maturation and may be transported to the nucleus from the cytoplasm. The transient nature of the nuclear fluorescence seen with Zika virus-infected cells suggests that the antigen might be synthesized early in the infectious cycle and then utilized in the cytoplasm at a later stage.
Involvement of the nuclear membrane during flavivirus infections is well recognized and the possibility that the nucleus is involved in flavivirus replication has been suggested (reviewed by Brinton, 1986). Thus far, using thin section electron microscopy, we have been unable to identify any virus-like nuclear structures in cells infected with Zika or LGT virus (M. Smith, unpublished results). This is not surprising since other morphogenetic studies have also failed to produce definitive evidence of such structures in the nucleus during flavivirus replication (reviewed by Murphy, 1980).

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