Electron Microscopic Immunoperoxidase Studies on the Accumulation of Virus Antigen in Cells Infected with Shope Fibroma Virus

By W. BOHN*

Bernhard Nocht Institute for Nautical and Tropical Medicine, Department of Virology, Bernhard Nocht-Str. 74, 2000 Hamburg 4, West Germany

(Accepted 10 September 1979)

SUMMARY

The indirect immunoperoxidase technique was used to investigate the development of the virus-specific intracellular and cell membrane antigens in cells infected with the Shope fibroma virus. Starting with 6 h p.i., virus antigen formed distinct inclusions within the cytoplasm frequently enclosed by endoplasmic reticulum. The endoplasmic reticulum disappeared almost completely 10 to 12 h p.i., coincidentally with the beginning of virus formation. The virus antigen was distributed throughout the cytoplasm. At the same time virus-induced antigen began to appear at the cell membrane and subsequently increased. No cytochemical staining could be observed on the endoplasmic reticulum, within the nucleus and within immature and mature virus particles. The correlation between antigen synthesis and changes in cell ultrastructure is discussed.

INTRODUCTION

Several ultrastructural studies have been carried out on the replication cycle of the Shope fibroma virus, a member of the genus Leporipoxvirus (Febvre, 1962; Scherrer, 1968a; Prose et al. 1971). They all showed the typical stages of poxvirus development, i.e. cytoplasmic inclusion bodies, immature spherical particles and their change to brick-shaped mature virions. Immunocytochemical studies with the light microscope have either shown virus-specific inclusion bodies in the cytoplasm (Kato & Cutting, 1959; Kato et al. 1963) or revealed the appearance of virus-induced antigen at the cell surface (Miyamoto & Kato, 1971; Tompkins et al. 1970; Singh et al. 1972). Ultrastructural immunocytochemical studies giving further evidence on the interaction between poxvirus antigen and cellular organelles have been limited by the lack of a proper method for the preservation of cell morphology. Cytochemical reactions were demonstrated only on mature virions (Morgan et al. 1961; Shabo et al. 1973) and single antigen inclusions (Thomas et al. 1974; Miyamoto & Nakane, 1976) with little relation to cell organelles. With the recent development of a saponin-aldehyde fixative (Bohn, 1978) a preparatory step was described allowing maintenance of cell structure and antigenicity as well as antibody penetration. With this technique and the immunoperoxidase method (Nakane & Pierce, 1966) the intracellular development of Shope fibroma virus-specific antigen was studied and correlated with virus-induced changes in cellular ultrastructure and the appearance of virus antigen at the cell membrane.

* Present address: Heinrich Pette-Institute for Experimental Virology and Immunology at the University of Hamburg, 2000 Hamburg 20, Martinistr. 52, West Germany.
METHODS

Cell culture. BHK-21 cells were grown on coverslips in Leighton tubes in Earle's minimum essential medium (MEM) supplemented with 10% foetal calf serum, 0.29% tryptose phosphate broth and Reofabin (200 µg/ml). Cells were grown to confluence and infected with 0.5 ml of stock virus at an input multiplicity of 0.5 to 1.0 rabbit 50% infectious doses (RID₅₀) per cell. After adsorption for 2 h at 37 °C the monolayers were rinsed with medium and incubated for up to 24 h. They were tested for virus antigen at hourly intervals beginning at 4 h p.i.

Virus. The Boerlage strain of the Shope fibroma virus (ATCC No. VR 253) was grown in rabbit testicles as follows. Each of four testicles was inoculated with 0.5 ml of the original virus suspension containing 10⁷ RID₅₀/ml. Seven days after infection, rabbit testicles were excised and homogenized with sterile sea sand in McIlvaine buffer, pH 7.2. The suspension was clarified at 1000 g, then layered on top of a 36% sucrose (w/w) solution and centrifuged at 50000 g for 90 min. The pellet was suspended in McIlvaine buffer and again centrifuged at 50000 g for 90 min. The concentrated virus was suspended in buffer and frozen in liquid nitrogen.

Infectivity assay. Purified stock virus infectivity was assayed by an in vivo test on domestic rabbits (Febvre, 1962; Verna & Eylar, 1962). Serial half-log dilutions (10⁻⁰.⁵) of stock virus were inoculated intracutaneously (0.1 ml per site) into the shaved flanks of adult rabbits. The rabbit infectious dose (RID₅₀) was calculated by the Reed & Muench (1938) method 8 to 10 days p.i., when the virus-induced tumours had reached their maximum size. Stock virus contained 10⁸.⁷⁵ RID₅₀/ml.

Antiserum. Antiserum against the Shope fibroma virus was prepared by inoculating rabbits intracutaneously with 1.0 ml of the stock virus solution, followed by an intramuscular booster injection with 1.0 ml stock virus 3 weeks later. After another 3 weeks the animals were bled, and sera were separated lyophilized and stored at -20 °C. For the immunocytochemical studies a fluorescein-conjugated (goat) anti-rabbit IgG (Miles Pentex, Israel) and a peroxidase-labelled (goat) anti-rabbit IgG (Miles Yeda Ltd., Rehovot, Israel) have been used. All sera were pre-adsorbed with uninfected cell monolayers.

Fixation and immunocytochemical staining. These procedures have been performed as described recently (Bohn, 1978). Briefly, for the demonstration of intracellular virus antigen, virus-infected cells were pretreated with a mixture of 0.05% saponin (Merck 7695, Darmstadt, Germany), 0.025% glutaraldehyde (Serva Feinbiochemica, Heidelberg, Germany) and 1% paraformaldehyde (Taab Laboratories, Reading, England) in 0.2 M-phosphate buffer (pH 7.3) for 5 min at 4 °C. They were rinsed briefly with buffer and postfixed with the previously mentioned fixative without saponin for 45 min at 4 °C. For the demonstration of cell membrane-associated antigen, saponin was not used in the fixative. Instead, fixation was done using 0.0125% glutaraldehyde and 1% paraformaldehyde in 0.2 M-phosphate buffer (pH 7.3) for 45 min. All subsequent washings were done with the previously mentioned phosphate buffer. After fixation cells were rinsed with buffer for 30 min at 4 °C, incubated with the anti-Shope fibroma serum for 45 min at room temperature, rinsed again with buffer for 45 min and treated with peroxidase-labelled antiserum for 45 min. The peroxidase substrate was made up freshly according to the method of Graham & Karnovsky (1966). Cells were incubated in the substrate solution for 10 to 15 min, rinsed with buffer, postfixed with 1% OsO₄, dehydrated with ethanol and embedded in Epon 812. Thin sections were studied without further staining unless otherwise stated.

Immunofluorescence studies were conducted on cells fixed with acetone for 10 min at -40 °C. Fixed cells were incubated with each of the two sera for 30 min at room temperature. Washing was done with the above mentioned buffer. Control procedures have been performed as described recently (Bohn, 1978) and did not show any staining.
Accumulation of Shope fibroma virus antigen

Fig. 1. Immunofluorescence of SFV-infected BHK-21 cells. (a) Six h p.i., distinct antigen inclusions within the cytoplasm; (b) 12 h p.i., antigen distributed throughout the cytoplasm.

RESULTS

Intracellular accumulation of virus-specific antigen

With the immunofluorescence technique performed on acetone fixed cells, the first virus-specific inclusion bodies could be observed 6 h p.i. (Fig. 1a). The dot-like sharply limited fluorescence was only found in the cytoplasm. Occasionally a weak staining area appeared around the nucleus. The amount of antigen increased with time after inoculation. Twelve hours p.i. virus-specific antigen was distributed throughout the whole cytoplasm (Fig. 1b) and 24 h p.i. nearly 30% of the cells contained inclusion bodies.

Using thin section electron microscopy, virus-specific antigen could first be detected at 6 h p.i. (Fig. 2a, b). Small antigen inclusions in the cytoplasm were partially surrounded by mitochondria and endoplasmic reticulum. The cytochemical reaction was restricted to the inclusion body. At a favourable plane of sectioning, the antigen inclusions could be visualized just below the cell membrane, which itself remained unstained (Fig. 2a). Virus antigen, which appeared at 8 h p.i., still formed distinct inclusions in the cytoplasm (Fig. 2c). The inclusions enlarged and at 10 h p.i. were sometimes completely surrounded by endoplasmic reticulum (Fig. 2d). Structures of virus particles were not detected until 10 h p.i. (Fig. 3a). From this time virus antigen began to lose its compact form and endoplasmic reticulum was hardly detectable. Furthermore, the sharp demarcation between cytoplasm and antigen inclusion began to disappear and virus antigen spread throughout the cytoplasm. Membranes of immature particles started to arise on the periphery of the antigen inclusions from distinct antigen complexes showing a strong cytochemical reaction. Twelve to 16 h p.i. virus antigen was distributed throughout the cytoplasm and the number of immature virus particles had greatly increased (Fig. 3b, c). At 16 h p.i. the first mature particles were observed (Fig. 3c). Twenty-four h p.i. the maturation of virus particles was complete in some cells. These cells showed a strong cytochemical reaction and the cytoplasm was completely vacuolated (Fig. 3d). Mitochondria were rounded up and no other intracellular membranes were visible. At no stage of virus development could any staining be observed either within the nucleus, the cisternae of the endoplasmic reticulum or at other intracellular membranes.
Fig. 2 to 4. SFV-infected BHK-21 cells stained for intracellular virus antigen with the indirect immunoperoxidase method. No heavy metal counterstain, unless otherwise stated. N, Nucleus; m, mitochondria.

Fig. 2. (a), (b). Six h p.i., distinct inclusions partially surrounded by endoplasmic reticulum; no staining at the cell membrane; (c) 8 h p.i., enlarged inclusions with a weakly stained centre; (d) 10 h p.i., inclusion completely surrounded by endoplasmic reticulum.

Cytochemical reaction on virus particles

Different section profiles of spherical immature particles demonstrated that in spite of the use of saponin, no cytochemical reaction occurred within closed immature particles (Fig. 4a). The interior of these particles looked completely homogeneous, showing a very weak contrast, whereas the surface was markedly stained. However, immature particles which were not closed also showed a staining reaction at the inside. At a higher magnification the trilaminar structure of the virus membrane could be discerned (Fig. 4b). As with
Fig. 3. (a) Ten h p.i., antigen has started to spread through the cytoplasm; the first virus membranes arose from distinct antigen complexes (arrows); (b) 12 h p.i., the first immature particles have appeared, mitochondria rounded up and the endoplasmic reticulum disappeared; (c) 16 h p.i. the first mature particles with strong cytochemical reaction appeared (arrow); (d) 24 h p.i., heavy staining throughout the vacuolated cell; mature virions (arrow).
immature particles, staining occurred not within mature particles, but only at their surfaces (Fig. 4d). In both cases heavy metal counterstain demonstrated the reasonable preservation of the internal virus structures (Fig. 4c, e).

**Antigen accumulation at the membrane**

At 10 h p.i. the first weak staining, distributed equally over the cell membrane of infected cells, appeared. At the same time, the first stages of virus synthesis were obvious. The intensity of staining remained unchanged up to 16 h p.i. and then increased in proportion to the number of virus particles (Fig. 5). Because saponin was not used in these experiments, intracellular virus particles did not show any immunocytochemical reaction (Fig. 5b). No staining was observed with a rabbit anti-vaccinia virus serum.

---

**Fig. 4.** Twenty-four h p.i., showing the immunocytochemical reaction on intracellular virus particles; (c) and (e) are counterstained with uranyl acetate. (a) Different section profiles of immature particles (arrows); the cytochemical reaction of closed particles occurs only at their surface; (b) shows the trilaminar structure of the virus membrane and the cytochemical reaction on both of its sides; (c) reveals the reasonable preservation of immature virus nucleoids (arrow); (d) cytochemical reaction of mature particles only at the surface; (e) nucleoids of mature virions are preserved (arrow).
Fig. 5. SFV-infected BHK-21 cells stained for cell membrane-associated, virus-specific antigen 24 h p.i.; no heavy metal counterstain; virus particles which have not reacted are indicated by arrows.

**DISCUSSION**

The results have shown that it is possible to study the intracellular development of pox-virus antigen during the replication cycle at an ultrastructural level. The relationship between the distribution of intracellular virus-specific antigen, the appearance of virus-induced antigen at the cell membrane and changes in cell ultrastructure could be demonstrated. Corresponding to our own observations, virus-specific inclusion bodies and virus-specific antigens were also observed as early as 6 h p.i. by Hodes & Chang (1968) using May-Grunwald-Giemsa-staining and immunofluorescence. That no virus antigen could be detected earlier than 6 h p.i. may be due to an inadequate quantity of antigens or to different antigenic properties of early and late proteins as was demonstrated for vaccinia virus proteins by Salzman & Sebring (1967).

With regard to the distribution of virus-specific antigen during the replication cycle, the results show a distinct parallel to the distribution of SFV-specific DNA as demonstrated by Scherrer (1968b). Autoradiography shows that DNA-marking was limited to the inclusion bodies, which became enlarged with time and reached a maximum intensity at 8 h p.i.
With the beginning of the virus formation at 10 to 12 h p.i., virus DNA was distributed throughout the whole cytoplasm.

Another aim was to show whether or not cell organelles take part in antigen synthesis. Sakaue & Kato (1974) indicated that some of the virus-specific proteins within cowpox virus-infected cells might be synthesized outside the inclusion body and transported into it. Thomas et al. (1974) observed electron-dense material within the cisternae of the endoplasmic reticulum of myxomavirus-infected cells, but could not reach it with immunocytochemical methods. In our own studies the participation of cellular organelles in virus antigen synthesis could not be confirmed. Virus antigen synthesis seemed to be limited to the inclusion bodies until their dissolution 10 to 12 h p.i. There was, however, a distinct correlation between virus antigen synthesis and changes in cellular ultrastructure. The most striking changes were associated with the endoplasmic reticulum, which progressively encircled the antigen inclusions up to 10 h p.i. This may be explained as being a process of displacing but may also be an active cellular process as it is known to be induced by some chemicals (Ghadially, 1975). The second striking change in cellular ultrastructure appeared at 10 to 12 h p.i. and coincided with the beginning of virus formation in that endoplasmic reticulum almost completely disappeared and virus antigen became distributed throughout the whole cytoplasm. It accounts for the beginning of cell degeneration at the start of virus formation.

In our studies, no labelling of antigen could be found within closed immature and mature virions despite the use of saponin. This may have been due to the failure of antibodies to penetrate the virions rather than to changes in antigenicity, because Thomas et al. (1974) were able to demonstrate internal labelling of myxomavirus particles.

Virus-induced antigens at the cell membrane could be detected as early as 10 h p.i. in agreement with the immunofluorescence observations by Miyamoto & Kato (1971) and Singh et al. (1972). With the macrophage migration inhibition test, virus-induced cell membrane antigens could be detected as early as 12 h p.i. by Tompkins et al. (1970). Endoplasmic reticulum seems to play no part in the synthesis and transport of SFV-specific antigen pre-dedined for cell membrane integration. Virus-specific antigen appeared to be associated with the membrane at a time when endoplasmic reticulum had almost disappeared and then increased with time. There would appear to be a correlation between cell degeneration, becoming obvious at the start of virus formation, and the appearance of cell membrane antigen. This agrees with the observations of an increased permeability of the cell membrane for soluble proteins after infection with SFV (Golubey et al. 1971), rabbit poxvirus (Schümpelki et al. 1978) and vaccinia virus (Ogier et al. 1974) at the beginning of virus formation. Further studies should establish whether cell membrane and intracellular antigens are identical. Monospecific antisera against structural components of the virus should provide information about the nature of the antigen observed in this study.

This work is part of a thesis in Biology at the University of Hamburg. The author is grateful to Dr. G. Nielsen for his advice and to Professor D. Peters for his critical and extensive review of the manuscript.

REFERENCES


Accumulation of Shope fibroma virus antigen


(Received 19 July 1979)