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AUTORADIOGRAPHIC STUDIES ON MOLLUSCUM CONTAGIOSUM USING ^{3}H-THYMIDINE[1]

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SUMMARY DNA synthesis in molluscum contagiosum nodules was investigated by autoradiography using ^{3}H-thymidine. Scratch preparations of molluscum nodule stained with Giemsa showed that many cells contained reddish purple cytoplasmic inclusions. These inclusions were classified according to their size and characteristics into stages I, II and III. The inclusions in stages I and II had the same staining characteristics as " B " type inclusions of poxviruses. Inclusions in stage II appeared to be gradually transformed into stage III inclusions, which had a lobular structure and corresponded to the so-called molluscum bodies of Lipshütz. The sites of cytoplasmic DNA synthesis corresponded exclusively with these inclusions. It was found by quantitative autoradiography that cells bearing inclusions did not show active nuclear DNA synthesis. Many cells with well-developed inclusions appeared to be degenerating. This suggests that virus-producing cells do not multiply but degenerate. Autoradiography of sections of the molluscum nodule showed that active nuclear DNA synthesis occurred only in the inclusion free-basal cells of the epidermis surrounding the molluscum foci. The host-virus interactions in molluscum contagiosum are similar to those in poxgroup viruses.

INTRODUCTION

Mollusum contagiosum is a relatively common skin disease in which small nodules appear in the human skin of infants, usually of 10 months to 3 years of age. In this disease, histological examination of the lesion shows a high degree of hyperplasia of prickle cells as multiple closely packed pearshaped lobules, and numerous inclusion bodies are seen in the epidermis. The inclusion bodies grow in size toward the surface of the skin. Evidence for the viral etiology of molluscum contagiosum is based on the presence of molluscum bodies in the epidermis of the lesion (GOODPASTURE *et al.* 1927: VAN ROOYEN, 1939) and of viral particles which can be seen by electron microscopy (GAYLORD *et al.* 1953; BANFIELD *et al.* 1959; SASAO 1959: FUJINAMI *et al.* 1963;

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LUTZNER 1963). The transmission of infection from one individual to another is occasionally observed clinically. However, the nature of molluscum contagiosum virus is still unknown since the isolation and *in vitro* propagation of the infectious agent have not yet been achieved.

LIPSCHÜTZ *et al.* (1919) measured the size of the elementary bodies in molluscum contagiosum and found large particles with diameters ranging from 300 to 350 mμ. RUSKA *et al.* (1943) demonstrated the brick-shaped outer shell of molluscum contagiosum virus by electron microscopy. Recently, EPSTEIN *et al.* (1966), using an *in vivo* autoradiographic method, reported a close relationship between DNA synthesis and molluscum contagiosum virus. The virus has provisionally been classified as a poxgroup virus on the basis of the size and shape of the particles (NOYES 1965 : Yaku 1966).

Poxgroup viruses generally have proliferative effects upon infected cells, and produce characteristic cytoplasmic inclusions which have been named after their discoverers. KATO *et al.* (1955, 1959, 1960 and 1963) found that these inclusions of poxviruses can be classified into two types, "A" and " B ", and that all poxviruses produced a common type of cytoplasmic inclusion, the " B " type, once viral multiplication occurred. The morphology, development, and staining characteristics of the " B " type inclusions of all the poxviruses are alike. Subsequently, by autoradiography and the fluorescent antibody technique, it became apparent that the sites of viral DNA synthesis and the sites of viral antigen in all poxviruses correspond exclusively to the " B " type inclusions. The "A" type inclusion, on the other hand, was found to play no role in the synthesis of virus materials.

In this way, poxviruses have a great advantage over other animal viruses as experimental materials, because cytoplasmic viral DNA synthesis is easily distinguishable from nuclear DNA synthesis of cells at the cellular level. Based on the unique characteristics of cytoplasmic viral DNA synthesis, KATO *et al.* (1962, 1965 and 1966) found that nuclear DNA synthesis was suppressed in cells showing multiplication of both oncogenic and non-oncogenic poxvirus. This suggests that poxvirus-producing cells do not multiply but degenerate.

This paper reports further investigations on the biological characteristics of inclusions of molluscum contagiosum and the relationship between viral multiplication and cellular proliferation in molluscum nodules.

MATERIALS AND METHODS

1. *Nodules of molluscum contagiosum*

Typical nodules of molluscum contagiosum of less than 2 to 3 mm in diameter were obtained from 25 patients, of between 10 months and 19 years of age. The method of collection and preservation of materials was as follows : the area of the lesion was disinfected with 70% ethanol and air-dried. The nodules were clipped off with a pair of sterile ophthalmic scissors. The specimens were immediately attached to the wall of a sterile test tube containing 1.0 ml of culture medium (90% of Eagle Minimum Essential Medium and 10% of calf serum) and were stored at 4°C.

2. *Scratch preparations of molluscum nodules*

Molluscum nodules were immersed in 0.25% trypsin solution and incubated at 37°C for 15 min. Then, scratch preparations were made on a cover slip, and were dried and fixed with methanol. Giemsa staining, or the Feulgen reaction were carried out on these samples.

3. *Autoradiography of sections of molluscum nodules*

Molluscum nodules were cultured in medium containing ^{3}H-thymidine (specific activity : 5 C/mM) at a final activity of 10 μc/ml for one to three hours at 37°C. Then the tissues were washed 3 times with Hanks' solution, fixed with Bouin's solution and embedded in paraffin and sections were made. After sectioning and removing the paraffin, samples were treated with 2% perchloric acid at 4°C for 40 min, and then washed with distilled water and dried. Dipping autoradiography was carried out using Kodak NTB 2 nuclear track emulsion. Samples were kept in a dark box containing silica gel at 4–6°C for

10–15 days. After exposure, the samples were developed, fixed and stained with hematoxylin-eosin.

4. *Autoradiography of scratch preparations of molluscum nodules*

Molluscum nodules were cultured in medium containing ^{3}H-thymidine for 1 hour. Then after trypsin treatment, scratch preparations were made as described above (3). The samples were dried and fixed with methanol, and treated with 2% perchloric acid at 4°C for 40 min. Dipping autoradiography was carried out on the scratch preparations of the nodules. Giemsa was used as a post-stain. The conditions of the radioactive isotope and autoradiographic procedures were as described above (3).

RESULTS

1. *Inclusion bodies in scratch preparations*

There were many cytoplasmic inclusion bodies which took on a reddish purple tinge in scratch preparations stained with Giemsa solution. These inclusion bodies varied from small, round, compact masses of less than 3 μ in diameter to large masses occupying the entire cytoplasm and pushing the nucleus aside (Figs. 1, 2, 3 and 4). Usually one and infrequently two inclusion bodies were seen within a cell. These inclusions were classified into 3 successive stages; stage I inclusion bodies were less than 10 μ in diameter, stage II were 10–30 μ in diameter, and stage III were more than 30 μ in diameter. The morphology and staining characteristics of stage I and II inclusion bodies were the same as those of " B " type inclusions of poxviruses. The size and structure of stage III inclusion bodies were very different from those of stage I and II bodies (Figs. 3 and 4). There were minute vesicular structures within the inclusion masses, which stained less with Giemsa than the surrounding material. Stage III type inclusion bodies have not been observed in other poxvirus infections, and probably correspond to the so-called " molluscum bodies ". Intermediate stages between stages II and III were seen, and the stage III bodies were considered as the most mature inclusions and differend from the "A" type inclusion bodies of the poxvirus group. The Feulgen reaction was positive in all stages.

2. *Autoradiography of scratch preparations*

A molluscum contagiosum nodule incubated at 37°C for an hour in culture medium containing ^{3}H-thymidine was trypsinized and a scratch preparation was prepared from it. Autoradiography was carried out on this. The sites of aggregation of silver grains in the cytoplasm corresponded exclusively to the inclusins (Figs. 5, 6 and 7).

Quantative comparisons were made of DNA synthesis in the nuclei and inclusion bodies of cells with and without inclusion bodies and in inclusions at different stages. Silver grain counts of nuclei were made on 90 randomly selected cells without inclusion bodies and 30 each of randomly selected cells with stage I, II and III inclusions. (Fig. 8).

Approximately 7% of cells showing no inclusion bodies exhibited intensive nuclear DNA synthesis. However, the number of silver grains in the nuclei in inclusion-bearing cells were none or far less.

The number of silver grains in the samples of 30 cells with inclusion bodies at different stages were compared with those in the nuclei of non-inclusion bearing cells. (Fig. 9). Intensive DNA synthesis was observed in all cells with inclusion bodies. The most intensive incorporation of ^{3}H-thymidine was found at atege II, with less at stage III. Many of the cells without inclusion bodies showed over 120 grains and probably contained over 200 per nucleus. The number of silver grains within the inclusion bodies also sometimes exceeded 120, but never exceeded 200.

The observation of active DNA synthesis within the nuclei of stage III inclusion-bearing cells may have been erroneous. This was becasue the silver grains in the inclusion bodies overlapped those in the nuclei. Nuclear DNA synthesis in cells with inclusion bodies never reached the level of nuclear DNA synthesis in cells without inclusions. (Figs. 9 and 10). The

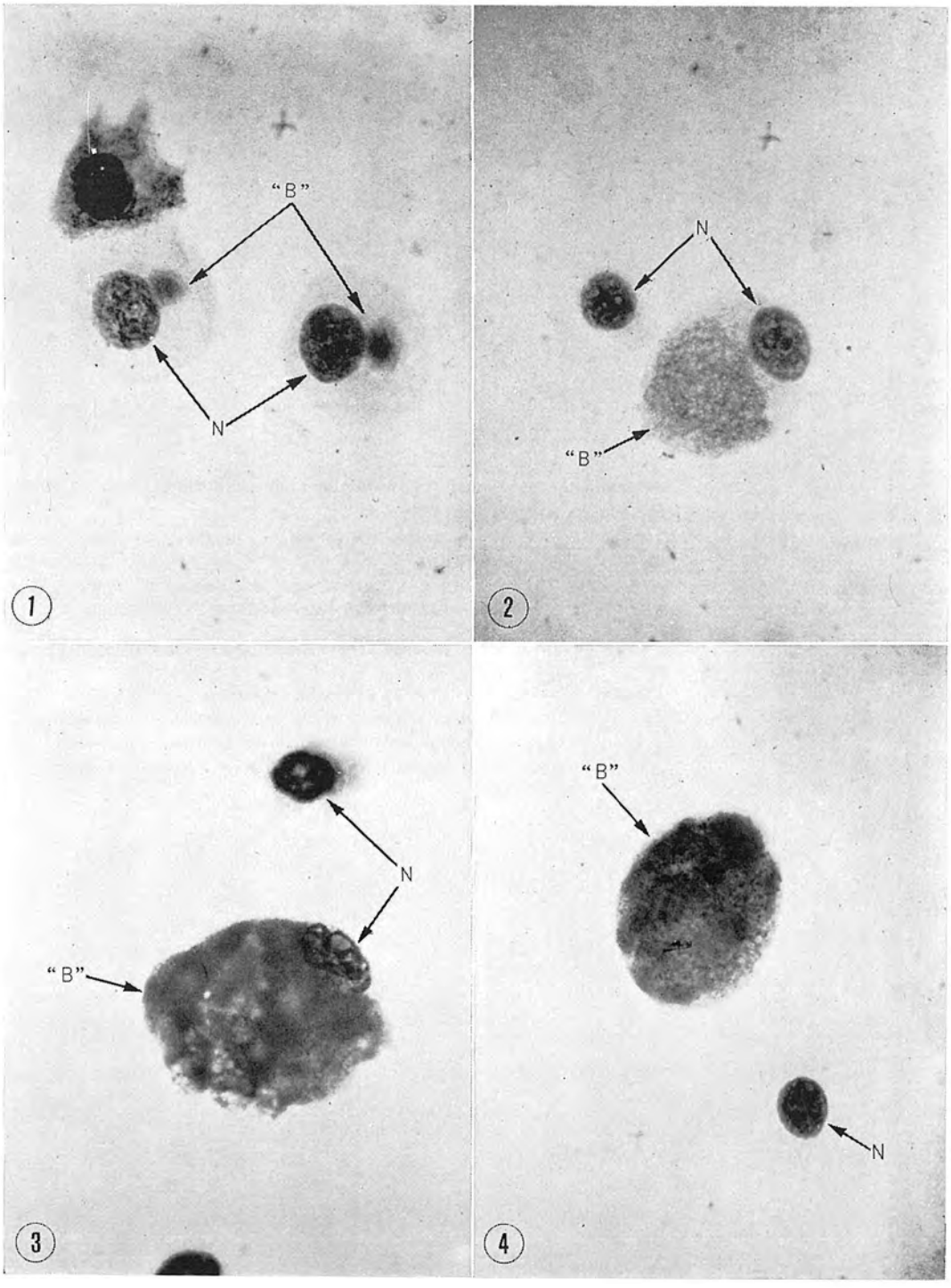
Scratch preparation of molluscum contagiosum stained with Giemsa. Azure II staining cytoplasmic inclusions are seen.

FIGURE 1 A round homogeneous cytoplasmic inclusion body in stage I. (×1200)

FIGURE 2 A granular cytoplasmic inclusion body in stage II. (×1200)

FIGURE 3 A huge amorphous cytoplasmic inclusion body in stage III. Note that the nucleus is pushed against the cell membrane. (×1200)

FIGURE 4 A huge amorphous cytoplasmic inclusion body in stage III. Note that the nucleus is not visible. (×1200)


"B"
N
1
N
"B"
2
N
"B"
3
"B"
N
4

Autoradiogram of scratch preparation of molluscum contagiosum. (Labelled for 3 hours with ^{3}H-thymidine).
Labelled " B " type inclusions are shown. Note that the nuclei of " B " bearing cells are not labelled.

FIGURE 5 Silver grains in stage I inclusion body. (×3000)

FIGURE 6 Silver grains in stage II inclusion body. (×3000)

FIGURE 7 Silver grains in stage III inclusion body. (×3000)

FIGURE 10 Comparison of DNA synthesis in the nuclei of cell without inclusions (above) and of an inclusion bearing cell (below). The silver grains are much less over the stage I inclusion body. (×1200)

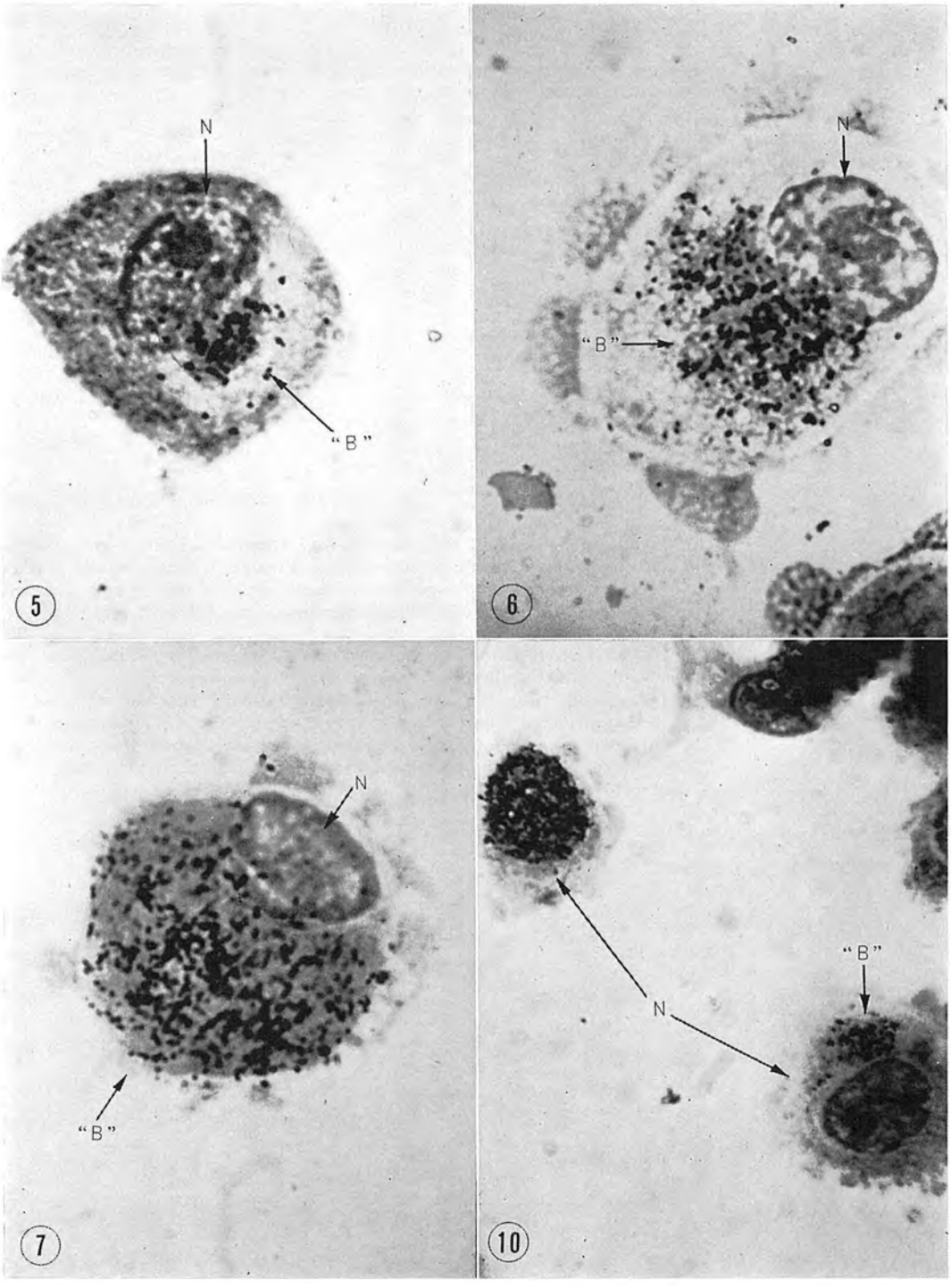
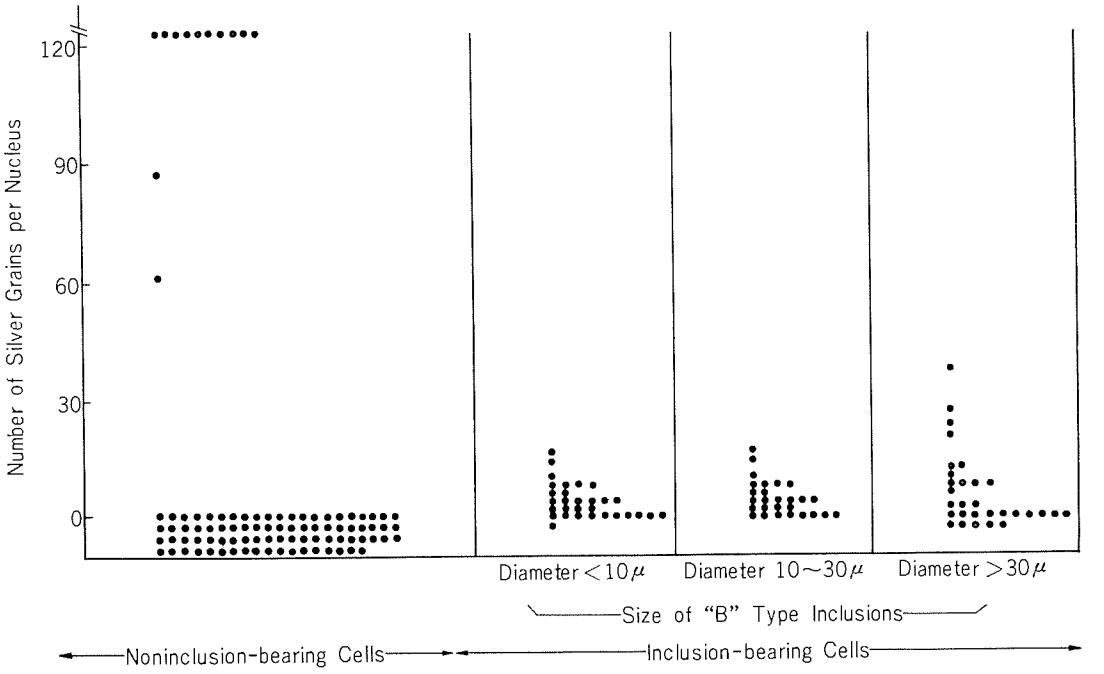
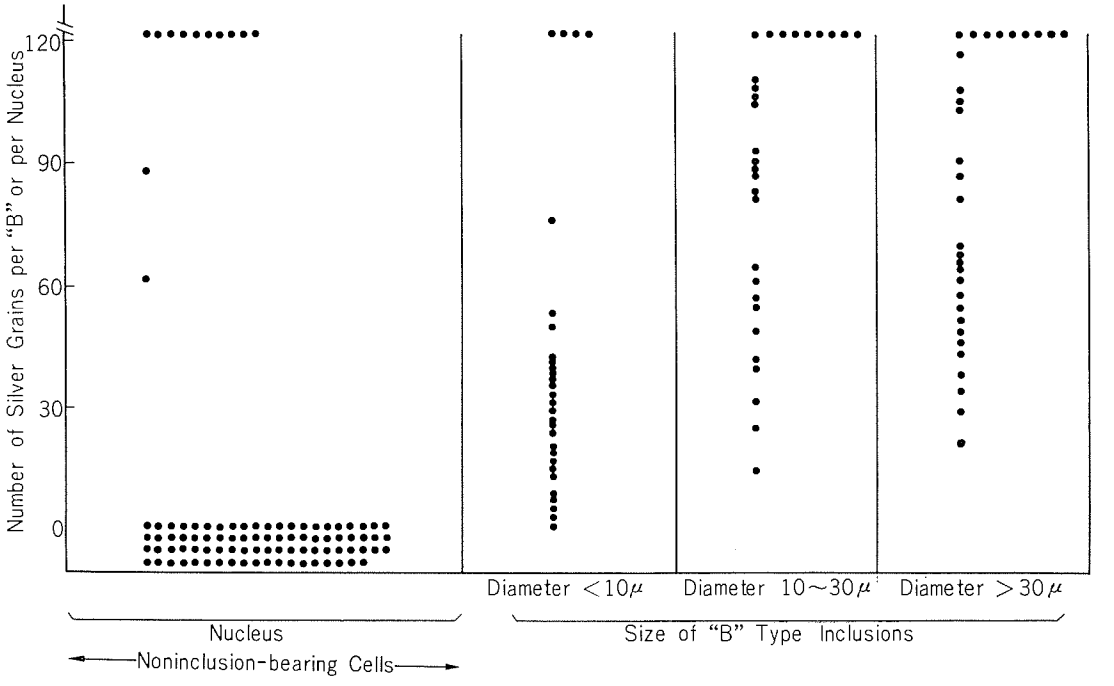
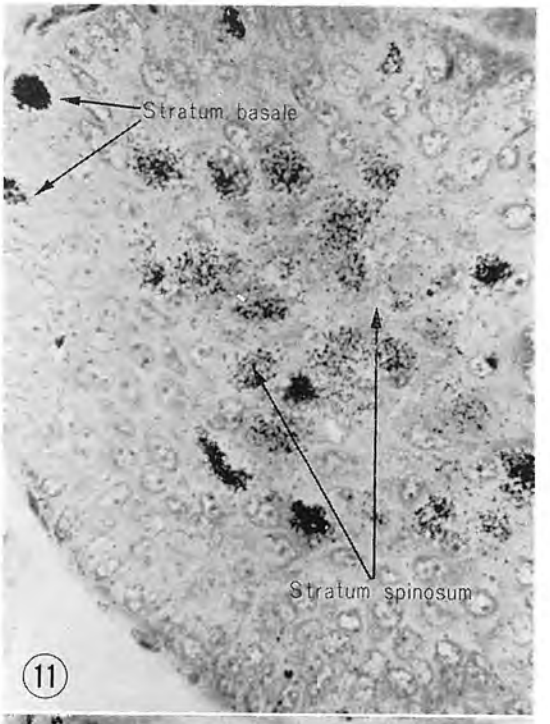

N
"B"
5
N
"B"
6
N
"B"
7
N
"B"
10

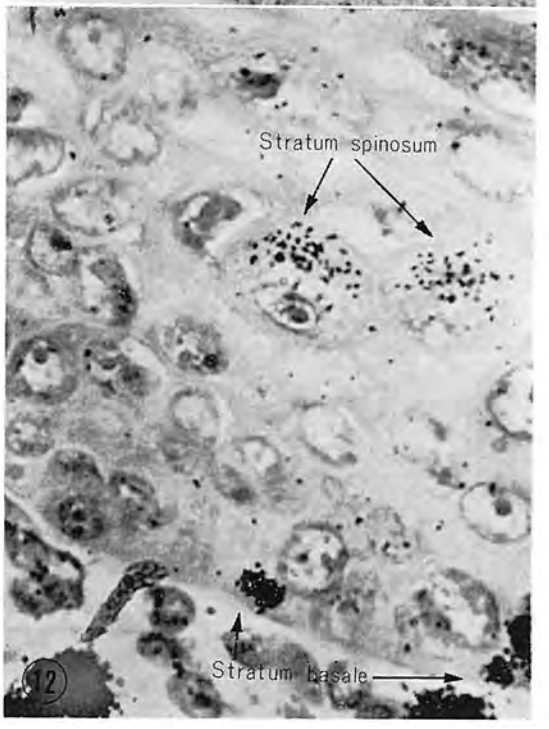
FIGURE 8 Effects of "B" type inclusion formation upon nuclear DNA synthesis. (1 hour preparation).
Inclusion bearing cells were divided into 3 groups according to the size of their inclusions. These cells were compared with cells containing no inclusion. The number of silver grains in the 30 labeled nuclei of each group are plotted. The dots above the level of 120 represent more than 120 silver grains.

FIGURE 9 Relationship between development of "B" type inclusions and their ability to synthesize DNA. (1 hour preparation).
Inclusion bearing cells were divided into 3 groups according to the size of their inclusions. These cells were compared with the cells containing no inclusions. The number of silver grains in the inclusions of the cells of each group are plotted. The dots above the level of 120 show more than silver grains.


Number of Silver Grains per Nucleus
120
90
60
30
0
Diameter < 10μ
Diameter 10~30μ
Diameter > 30μ
Size of "B" Type Inclusions
Noninclusion-bearing Cells
Inclusion-bearing Cells


Number of Silver Grains per "B" or per Nucleus
120
90
60
30
0
Diameter < 10μ
Diameter 10~30μ
Diameter > 30μ
Nucleus
Size of "B" Type Inclusions
Noninclusion-bearing Cells





Autoradiogram of a lobule of mollusucum contagiosum cultivated *in vitro* after incubation in medium containing 10 μc/ml ^{3}H-thymidine.

FIGURE 11 Silver grains are found throughout the whole epidermal layer, especially in cells of the stratum basale and lower stratum spinosum. (×600)

FIGURE 12 Distribution of silver grains in the strata basale and spinosum. Note the difference in density and number of grains in the basal and prickle cells. (×1200)

nuclei of cells with inclusion synthesized DNA slowly and appeared to be partially degenerating and these nuclei never proliferated.

3. *Autoradiography of histological sections*

Sections of mulluscum nodules were autoradiographed after incubation at 37°C for 1–3 hours in culture medium containing ^{3}H-thymidine. Control autoradiographs of normal human skin were also made following the same procedure. DNA synthesis in normal human skin was limited to a few nuclei of the stratum basale and the neighbouring stratum spinosum cells, while in the molluscum contagiosum nodule, cytoplasmic DNA synthesis occurred in groups of cells in the stratum spinosum (Fig. 11). As a rule, in cells located in the center of a focus, silver grains were diffusely scattered in the cytoplasm, corresponding to the " molluscum bodies ". On the contrary, most of the cells located in the outer parts of a focus contained small localized aggregates of silver grains which corresponded to the early stage of inclusion bodies. This peculiar arrangement of inclusion-bearing cells in a focus suggests that the infection starts at the center of the focus and progresses radially to the peripheral part of the focus.

Nuclear DNA synthesis was greatly diminished in cells with active cytoplasmic DNA synthesis. Prominent nuclear DNA synthesis was observed mainly in the basal layer of the epidermis. (Fig. 12).

DISCUSSION

1. *Inclusion bodies of molluscum contagiosum*

Isolation of the molluscum contagiosum virus has not been achieved, but the virus has been classified as poxgroup virus on the basis of its morphology.

Since LIPSCHÜTZ reported on the inclusion bodies of molluscum contagiosum in 1919, these bodies have been studied by many investigators mostly in sections of the nodules. The molluscum body contains abundant DNA and is separated into small loculi by trabeculae which are considered to be made of RNA (RAKE *et al.*, 1950). These observations on the molluscum body agree with the present findings.

KATO *et al.* (1955, 1959, 1960 and 1963) studied the inclusion bodies of the poxvirus group extensively, and classified them into "A" and " B " types. The " B " type inclusion bodies, which are found in cells infected with poxviruses, all have the same staining properties with Giemsa stain and the same morphological developmental process. In all poxvriruses, the inclusion bodies have been shown to be the site of synthesis of viral DNA and of viral antigen by autoradiography using ^{3}H-thymidine and the fluorescent antibody technique.

If molluscum contagiosum virus belongs to the poxgroup virus, then its inclusions should have morphological similarities to " B " type inclusion bodies. However, as far as the molluscum bodies are concerned, the inclusion bodies had rather different structures from " B " type inclusion bodies although DNA synthesis was observed in them. In our present experiments, various size of cytoplasmic inclusion bodies were found in many epidermal cells of molluscum nodules and these stained reddish purple with Giemsa (Figs. 1, 2 and 3). These inclusion bodies were tentatively divided into 3 successive stages on the basis of their size and properties. The staining and structural properties of stage I and II inclusion bodies corresponded to " B " type inclusion bodies of poxgroup viruses, while stage III inclusion bodies of more than 30 μ in diameter corresponded to the so-called " molluscum bodies ". There were intermediate stages between stage II and III, and stage III inclusion bodies were shown to be specific molluscum inclusion bodies in the developmental process. Therefore, stage III inclusion bodies are different from the "A" type inclusion bodies of poxgroup viruses which develop independently from the " B " type inclusion bodies. Thus the inclusion bodies of molluscum contagiosum showed a similar developmental process to the " B " type inclusion bodies of poxviruses but were finally transformed into the

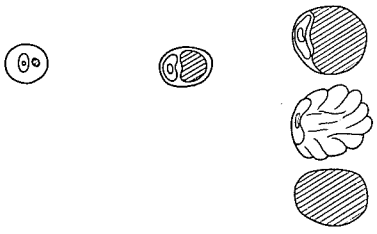
		Molluscum contagiosum virus infected cell			Poxvirus infected cell	
		Stage I	Stage II	Stage III	"B"	"A"
Structure of infected cells						
Size		10 μ	10–30 μ	30 μ		
Changes of cell structure	Nucl.	no changes	Enlargement of nucleolus. Compression of nucleus against the cell membrane	Atrophy and disappearance of nucleolus. Compression of nucleus against the cell membrane		
	Incl.	homogeneous compact	homogeneous minute granule	homogeneous lumpy	compact diffuse homogeneous granule	compact homogeneous
Method	Fixative					
Giemsa	MtOH	reddish	bright-red	reddish purple pale	reddish purple	pale blue
IN HCl 60°C 5 min.+Giemsa	MtOH	+	+	+	+	–
H. E.	MtOH	brownish purple	brownish purple	pale brown	pale	red
Feulgen	MtOH	+	+	+	+	–
Autoradiography of ^{3}H-thymidine	MtOH	+	+	+	+	–

FIGURE 13 Comparison of morphological characteristics of inclusions of molluscum contagiosum and poxvirus. (MtOH: Methouol)

" molluscum bodies ".

An autoradiography with ^{3}H-thymidine, the site of agggregation of silver grains in the cytoplasm corresponded to inclusion bodies at all these stages (Figs. 5, 6 and 7). Silver grains were even found in stage III inclusion bodies which spread through the entire cytoplasm and pushed the nucleus against the cell membrane (Fig. 7).

The number of silver grains in inclusion bodies at all stages were far fewer than in nuclei of cells without inclusion bodies (Figs. 8 and 9). In cells infected with cowpox virus, DNA synthesis in " B " type inclusion bodies, and especially those of 10 to 20 μ in diameter, was as active as that in the nuclei of cells bearing no inclusion bodies. Thus, it seems that the cytoplasmic DNA synthesis of the molluscum

contagiosum inclusion bodies occurs at a much lower rate, and this cytoplasmic DNA synthesis could be considered as evidence for viral DNA synthesis.

2. *Relationship between viral multiplication and cell proliferation in molluscum contagiosum nodule*

On autoradiography of ^{3}H-thymidine uptake using scratch preparations, intensive labelling of the cytoplasmic inclusion bodies was seen (Figs. 5, 6 and 7). There was much less labelling of the nuclei than of nuclei of cells without inclusions (Figs. 9 and 10). Among these inclusion-bearing cells, which did not show any active nuclear DNA synthesis, the cells with stage III inclusion bodies appeared to be degenerating. This suggests that virus-producing cells do not proliferate. A similar suppression of nuclear DNA synthesis in cells in which there is viral DNA synthesis has also been observed with other poxviruses.

In autoradiograms of sections of molluscum nodule, cytoplasmic DNA synthesis was seen in groups of cells in the stratum spinosum (Fig. 11). The structure of a focus was quite similar to that of the foci found in rabbit corneal cells infected with vaccinia virus (HARA *et al.* 1964), and indicated a centrifugal progression of the infection. Nuclear DNA synthesis in cells with active cytoplasmic DNA synthesis was rarely seen as in scratch preparations (Fig. 12). Prominent nuclear DNA synthesis was mainly observed in the basal layer of the epidermis (Figs. 11 and 12). These facts suggest that formation of small tumors in the molluscum contagiosum cannot be ascribed to division of virus producing cells, but to proliferation of cells of the stratum basale and the neighbouring stratum spinosum.

The host-virus interaction in the formation of the nodules of the molluscum contagiosum strongly resembles to that of other poxgroup viruses, in regard to the characteristics of the inclusion bodies and to DNA synthesis (Fig. 13).

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