Cytochemistry of Virus-Induced Inclusions Containing Nucleic Acids[1] (35430)

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Accumulations of viral RNA or cellular inclusions produced by riboviruses contain RNA demonstrable by current cytochemical staining reactions for RNA, such as acridine orange (A) (1), toluidine blue, and toluidine blue-molybdate (TBM) (2–6). Staining of this viral RNA by these methods can be prevented for the most part by previous digestion with ribonuclease (RNase). Accumulations of RNA that stain red with AO in cells infected with reovirus (7), poliovirus, mengovirus, and influenza virus (1) are susceptible to digestion with RNase. Similarly, the staining of inclusions produced by rabies virus (3, 4) parainfluenza virus No. 3 (5) and by influenza virus (6) with toluidine blue and toluidine blue-molybdate can be prevented by digestion with RNase. However, a discrepancy has been observed between the effects of nuclease digestions on the staining properties of a number of virus-induced inclusions by AO (1) and by other methods of staining (3, 4, 6, 8, 9). The substance responsible for green staining by acridine orange is not susceptible to digestion by deoxyribonuclease (DNase) (8) or by RNase (3, 4). Thus, AO staining of the inclusions produced by SV40 virus, which contain double-stranded DNA (10), is not affected by digestion with DNase (1, 8, 11). On the other hand, staining of SV40 inclusions by toluidine blue, toluidine blue-molybdate, pyronin methyl green or by the Feulgen procedure is prevented by digestion with DNase (8). In spite of the fact that unencapsidated viral nucleic acid is present in large amounts, it has been claimed that the DNA of such viruses as adenovirus, herpes, and polyoma can be distinguished from cellular DNA by virtue of the fact that it is only susceptible to DNase digestion after proteolytic enzyme digestion (1).

To clarify this problem, the cytochemical properties of the double-stranded RNA inclusions induced by reovirus were examined and the nature of the green staining of virus-induced inclusions by AO is reviewed in the light of previous experiments with inclusions containing DNA (8, 9) and RNA (3–6). The effects of various extraction and digestion procedures on staining of these inclusions by AO and by other staining procedures suggest that, in these systems, green staining by AO is not due to binding of the dye by viral nucleic acids.

Material and Methods. Virus. Reovirus, type 3 was kindly provided by Dr. Angus Graham.

Infected cells. L cells were grown on coverslips at 37° in Eagle's basal medium containing 2 m*M* glutamine and 10% filtered calf serum with added bicarbonate (5.6% $NaHCO_3$, 25 ml/liter) and chlortetracycline (50 μg/ml) in a humidified atmosphere containing approximately 5% CO_2. Cells were infected with a high multiplicity of virus and examined 36 to 48 hr later when inclusions of all sizes from barely detectable granules to large bodies surrounding the nucleus were present (Fig. 1).

Cytochemistry. The cytochemical techniques used to demonstrate nucleic acids, proteins, lipids, and mucopolysaccharides were essentially those described in a previous publication (3). Cells were stained by the toluidine blue-molybdate, method C, which de-

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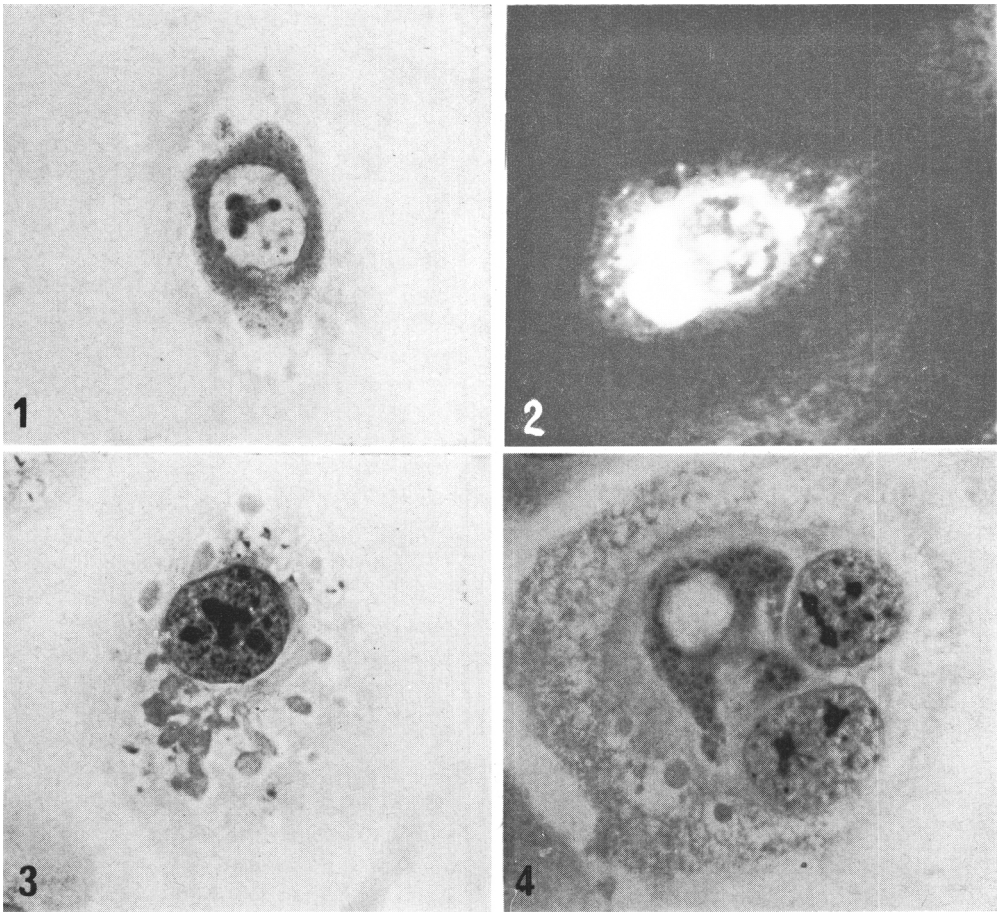

FIG. 1. Large inclusion body surrounding the nucleus; purple granules are embedded in a less intensely stained matrix. TBM, method A, ×1100.

FIG. 2. Intensely green-staining inclusion in cell after extraction of nucleic acids by hot trichloroacetic acid; acridine orange, ×1100 (print from Ektachrome color transparency).

FIG. 3. Metachromatically stained inclusions; TBM, method C, ×1100.

FIG. 4. Bluish-purple granular inclusions stained by dilute toluidine blue after nitrosation; ×1100.

pends upon the unmasking of nucleic acid phosphoryl groups by deamination of protein-bound amino groups. The nucleic acid phosphoryl anions bind the cationic dye, toluidine blue, and the intensity of staining is increased by a polymerization of the dye molecules by ammonium molybdate. For acridine orange (AO) staining, preparations were fixed for 15 min in Carnoy's fluid and stained for 8 min with 0.01% acridine orange in McIlvaine's buffer at pH 3.5. Preparations stained at pH 3.0, 3.5, and 4.0 were found to be indistinguishable. The pH of 3.5 was selected because this had been used in previous work (3). The specificity of staining of nucleic acids was checked by digestion with RNase and/or DNase (12) with or without treatment with pepsin (0.2% in 0.02 *N* HCl (1). (The pepsin, obtained from the Nutritional Biochemical Company, was 3 times crystallized). Nucleic acids were also removed by treatment for 30 min in 5% trichloroacetic acid (TCA) at 90° for 30 min (13).

Results. The staining properties of the cytoplasmic inclusions are summarized in Table I. The yellow green staining of the inclusions by AO was unaffected by digestion with nucleases or by extraction of nucleic acids

TABLE I. Staining Properties of Reovirus-Induced Cytoplasmic Inclusions.[a]

Pretreatment before staining	Staining procedures[b]				
		TBM; methods:			
	AO or HNO_2AO	A, B, and E	C	Dilute TB after HNO_2	Pyronin–methyl green
None or incubation in buffer or DNase	Yellow green 2+	Purple granules in blue or purple matrix	Purple + to 2+	Blue to purple + to 2+	Red 2+
RNase alone or preceded or followed by DNase digestion	Yellow green 2+	0	0 or +[c]	0	0 to +
TCA 90°, 30 min	Yellow green 2+	0	0	0	0 to trace

[a] Abbrev.: AO = acridine orange; TBM = toluidine blue-molybdate; TB = toluidine blue; TCA = trichloroacetic acid; RNA = ribonuclease; DNase = deoxyribonuclease.

[b] 0–2+ indicates relative intensity of staining, 0 being no stain.

[c] No stain if digested with RNase after HNO_2 treatment. Stained if digested before HNO_2.

by hot TCA (Fig. 2). Staining of the cellular DNA or RNA by AO was abolished by digestion with the appropriate nuclease and, with the exception of the inclusions, practically all staining of the cells was prevented by extraction of both nucleic acids by hot TCA. In preparations stained by toluidine blue-molybdate (TBM) methods A, B, and E the inclusions appeared to consist of purple granules embedded in a homogeneous matrix that was orthochromatically stained when the inclusions were small, and purple when they were large (Fig. 1). The inclusions were stained purple by TBM, method C, the larger ones being more intensely stained (Fig. 3). The smaller inclusions were stained orthochromatically, and the larger metachromatically by dilute toluidine blue (TB) after nitrosation (Fig. 4). The inclusions were stained red in preparations stained with pyronin–methyl green. Digestion with RNase prevented all staining of inclusions by TB and the TBM methods, with one exception. If the RNase digestion followed the nitrosation stage in TBM, method C, the inclusions were unstained. If, on the other hand, the digestion preceded the nitrosation, the inclusions could still be weakly stained. Removal of nucleic acids by hot TCA resulted in no staining of inclusions, or cells, by TB and TBM. The affinity of the inclusions for pyronin was largely removed by digestion with RNase or hot TCA extraction. The staining of inclusions by all methods was unaffected by digestion with DNase or the Tris buffer (12) in which the enzymes were dissolved.

In view of the possibility that resistance of AO staining material in the inclusions to digestion with RNase was due either to the presence of double-stranded RNA or to masking of nucleic acid phosphoryl groups by protein, preparations were treated with HNO_2 as for TBM, method C (2), and then stained with acridine orange. The inclusions were stained yellowish green as with the regular acridine orange method, unlike the chromatin which was stained red. Treatment with RNase and/or DNase had no effect on the staining of inclusions by AO after nitrosation.

Digestion with pepsin alone or in combi-

nation with nucleases proved very destructive to the cells especially if treatment with pepsin preceded the nuclease digestion. For the most part, pepsin with or without RNase or DNase, or 0.2 *N* HCl alone removed the inclusion bodies leaving holes in the cytoplasm although, occasionally, weak green staining inclusions persisted. The effects of these procedures were variable in different areas of each preparation and from one experiment to another.

Protein-bound groups of the inclusions were stained by the Morel Sisley reaction (14), a modification of the Pauly method for histidine (15), the dinitro-fluorobenzene reaction (16) the Ninhydrin Schiff method. The inclusions were not stained by the alkaline fast green method for histones (17). The inclusions contained no lipid demonstrable by Sudan black staining and no acid mucopolysaccharides stainable by the periodic acid–Schiff method or by alcian blue (13).

Discussion. The results clearly indicate that reovirus-induced inclusions contain RNA that is stainable by dilute TB after nitrosation, by 4 different TBM methods and by pyronin. RNA that is stained by these methods is predominantly single stranded, since it can be digested by RNase. The effects of RNase digestion on staining of inclusions by TBM, method C, suggests that both single- and double-stranded RNA are responsible for dye binding. Digestion with RNase before treatment with HNO_2 completely prevented all staining of cellular RNA, but did not abolish the affinity of the inclusions for stain, presumably because the double-stranded RNA was resistant. Hot TCA removed all stainable material from the inclusions, indicating that the RNase-resistant material was nucleic acid. On the other hand, when the cells were digested with RNase after nitrosation and deamination by HNO_2 the inclusions could no longer be stained by the TBM method. Presumably the deamination of bases (18) leads to disruption of hydrogen bonding of the RNA duplex with resultant increase in susceptibility to digestion by RNase.

Staining of reovirus inclusions by acridine orange does not appear to be due to any detectable binding of the dye by RNA, since it was unaffected by digestion with ribonuclease preparations that effectively diminished or abolished staining by other methods. The complete resistance to RNase digestion cannot be attributed to the presence of RNA in the double-stranded form since some single-stranded RNA is present in reovirus (19), and during virus replication each double-stranded genome RNA segment is transcribed into single-stranded mRNA (20). Also, deamination of the bases by nitrous acid resulted in a change from green to red staining of chromatin by AO, presumably as a result of denaturation of DNA, while this treatment did not affect the staining of reovirus inclusions. Furthermore, staining with AO was unaffected when all forms of RNA were removed by hydrolysis with hot TCA. While acridine orange stains the nucleic acids of the cell, it does not appear to induce any detectable staining of double- or single-stranded reovirus RNA. In other experiments reported elsewhere (3, 4, 8), AO did not stain the RNA of rabies virus inclusions nor the DNA of SV40 virus inclusions. It has been suggested that viral nucleic acid is resistant to digestion with nucleases because it is protected from this action by the protein of the capsid (1). This seems improbable because much of the nucleic acid of viral inclusions is not encapsidated. Furthermore, nucleases are quite effective in removing both RNA and DNA that is stainable by many other procedures. Thus, the Feulgen-positive DNA of SV40-induced inclusions was completely digested by DNase (8). Similarly, the RNA of rabies virus inclusions that is stainable by toluidine blue and TBM was readily digested by RNase while the green staining by AO was unaffected by either RNase or hot trichloroacetic acid extraction (3, 4). Furthermore, the nucleic acids of inclusions in cells infected with polyoma (21), herpes (9), influenza (6), and parainfluenza (5) were also susceptible to digestion with nucleases. While the nucleic acids of intact viruses are protected from the action of nucleases (22, 23), viral RNA (24) and DNA (25) are susceptible to digestion after suitable fixation.

The results of treatment of cells with hydrochloric acid, pepsin, or combinations of

pepsin and nucleases were just as unsatisfactory as in previous experiments with rabies and SV40 inclusions (3, 4, 8). Damage to cells was so great and so variable following any one of these procedures that no meaningful conclusions could be reached. Certainly, when inclusion bodies, as well as other areas of the cytoplasm, were removed by pepsin treatment these areas could no longer be stained. This does not, however, imply the presence of any particular nucleic acid at the sites of digestion.

Since the reovirus inclusions did not stain with toluidine blue after removal of nucleic acid and were not stained by alcian blue or by the PAS method, staining by AO cannot be attributed to mucopolysaccharides. Similarly, mucopolysaccharides could not be detected in rabies and SV40 inclusions (3, 4, 8). All three types of inclusions could be stained by various methods for protein-bound groups. It would appear, therefore, that the green staining by AO is attributable to binding of dye by protein-bound carboxyl groups. It has been shown that, under certain conditions, basic dyes are bound by protein as well as by nucleic acids above pH 4.2 (26). However, the pH at which basic dyes will bind the carboxyl groups of protein will depend upon the type of fixation (27), concentration of dye, temperature, and many other factors (28). Thus the dye, AO, rather than the nuclease does not appear to have access to viral nucleic acid phosphoryl groups in the inclusions described here, presumably because it fails to penetrate the proteins of the capsid. One other possibility is that AO does stain the viral nucleic acids but that such staining is obscured by the much more intense staining of protein.

Summary. Cytoplasmic inclusions produced by reovirus, type 3, contain RNA that is stainable by 4 TBM methods, by dilute toluidine blue after nitrosation and by pyronin. Staining of inclusions by all these methods, except for TBM, method C, is due to the presence of single-stranded RNA which is susceptible to digestion by RNase. Double-stranded RNA is partially responsible for staining by TBM, method C, since staining is reduced but not abolished by digestion with RNase. Hydrolysis of RNA by hot TCA prevents all staining by these methods. The ability of the reovirus inclusion to stain green with acridine orange is unaffected by all methods that remove nucleic acids demonstrable by other methods and is probably due to dye-binding by protein. The results of digestion by combinations of nucleases and pepsin are variable, irreproducible and incapable of logical interpretation.

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