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Double-Stranded RNA Is Produced by Positive-Strand RNA Viruses and DNA Viruses but Not in Detectable Amounts by Negative-Strand RNA Viruses

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Double-stranded RNA (dsRNA) longer than 30 bp is a key activator of the innate immune response against viral infections. It is widely assumed that the generation of dsRNA during genome replication is a trait shared by all viruses. However, to our knowledge, no study exists in which the production of dsRNA by different viruses is systematically investigated. Here, we investigated the presence and localization of dsRNA in cells infected with a range of viruses, employing a dsRNA-specific antibody for immunofluorescence analysis. Our data revealed that, as predicted, significant amounts of dsRNA can be detected for viruses with a genome consisting of positive-strand RNA, dsRNA, or DNA. Surprisingly, however, no dsRNA signals were detected for negative-strand RNA viruses. Thus, dsRNA is indeed a general feature of most virus groups, but negative-strand RNA viruses appear to be an exception to that rule.

Double-stranded RNA (dsRNA) of more than 30-bp length is a key activator of the innate immune response against viral infections (2, 8, 25, 50, 61, 66). The interaction of the host cell with dsRNA occurs by several mechanisms. Specific receptors activate the synthesis of antiviral type I interferons (IFN-α and IFN-β) and antiviral proteins (20), and dsRNA-activated enzymes can directly inhibit viral replication (50, 55, 70). The RNA helicases RIG-I (72) and MDA-5 (4) as well as the protein kinase PKR (70) bind to intracellular dsRNA and lead to the activation of the transcription factors interferon regulatory factor 3 (IRF-3) (51, 67, 73) and NF-κB (31, 74), respectively, which are important for IFN synthesis. Toll-like receptor 3 (TLR3) binds to extracellular and endosomal dsRNA and also activates IFN transcription via IRF-3 and NF-κB (3, 46, 61). In addition, IFN effector enzymes, such as PKR (70), 2′-5′-oligoadenylate synthetase (2-5-OAS) (55) and the RNA-specific adenosine deaminase (50), need to be activated by dsRNA and inhibit viral replication at various levels. Viruses, in turn, escape this immune response either by expressing dsRNA-binding proteins or by other strategies to inhibit the dsRNA-induced pathways (18, 25, 68).

Clearly, both host and viral pathogens apply a range of measures to deal with dsRNA, indicating that this molecule represents a danger signal of central importance for the innate immune response. It is widely assumed that dsRNA is generated by viral RNA polymerases either as an intermediate in genome replication (RNA viruses) or as an erroneous product due to converging bidirectional transcription (DNA viruses) (25, 32). However, to our knowledge, this has been directly shown for only a few viruses (33, 57, 69), whereas in most cases, only indirect evidence, such as activation of the dsRNA-dependent enzymes PKR, 2-5-OAS, and RNA-specific adenosine deaminase (28, 50, 70), activation of TLR3 (60), and investigation of the dsRNA content of cellular lysates (25, 32, 40), is available.

In this study, we attempted to find evidence for virally produced dsRNA in situ, using a nondestructive method. By employing a dsRNA-specific antibody for immunofluorescence analysis, we investigated the presence and localization of dsRNA in cells infected with a range of viruses. Indeed, we detected significant amounts of dsRNA for viruses with a positive-strand RNA or a dsRNA genome as well as for DNA viruses. Surprisingly, however, no significant dsRNA signals were detected for negative-strand RNA viruses, suggesting that other viral components are more important in triggering the host's antiviral response.

MATERIALS AND METHODS

Cells and viruses. Vero cells, HeLa cells, and BHK-1 cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The viruses used were adenovirus type 5 (AdV), encephalomyocarditis virus strain FA (EMCV), influenza A virus strain PR8 (FLUAV), herpes simplex virus 1 (HSV), La Crosse virus (LACV), modified vaccinia virus strain Ankara (Vac), reovirus strain Lang (ReoV), and severe acute respiratory syndrome coronavirus strain FFM (SARS-CoV). AdV and Vac were propagated in HeLa and BHK cells, respectively, while the other viruses were propagated in Vero cells.

Enzymes. RNase III was supplied by Ambion and RNase A by Sigma-Aldrich. Both enzymes were incubated for 2 h at 37°C in their respective reaction buffers provided by the manufacturers.

Poly(I:C) transfection. For transfection of cells with synthetic dsRNA, 10 μg of poly(I:C) (Sigma) was prepared with 10 μl of Metafectene (Biontex) in 200 μl of serum-free medium according to the manufacturers' instructions. After 15 min of incubation, the dsRNA-liposome mixture was dropped onto cells using the same medium.

Immunofluorescence analysis. Cells were grown on coverslips to 30 to 50% confluence and transfected or infected as indicated. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.5% Triton X-100 dissolved in phosphate-buffered saline (PBS). For dsRNA immunofluorescence, the mouse mono-

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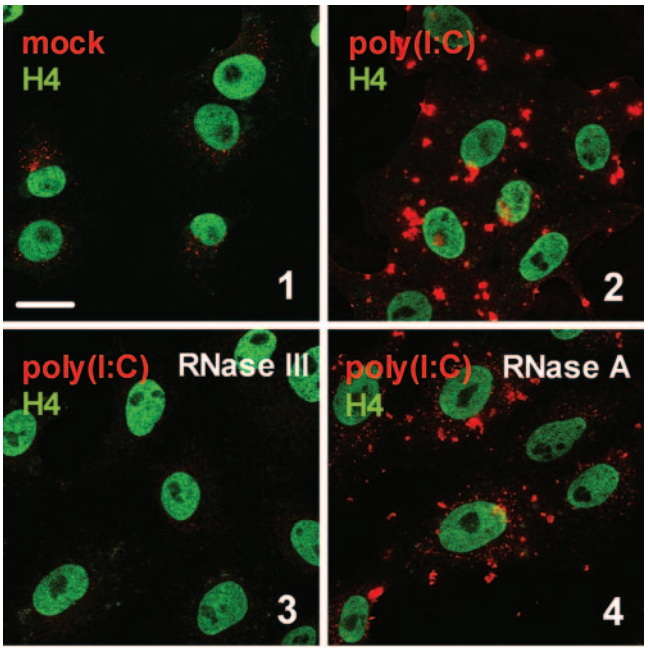

FIG. 1. The monoclonal antibody J2 specifically recognizes dsRNA. Vero cells were transfected with poly(I:C) as indicated in Materials and Methods. After an incubation period of 6 h, the cells were fixed and analyzed for dsRNA using the mouse monoclonal antibody J2 (red). To visualize the cell nuclei, histone H4 was stained using a specific rabbit antiserum (green). Shown are untransfected cells (1), poly(I:C)-transfected cells (2), and poly(I:C)-transfected cells treated with either 2 U of RNase III (3) or 2 U of RNase A (4). Bar, 20 μm. All pictures were taken with the same magnification.

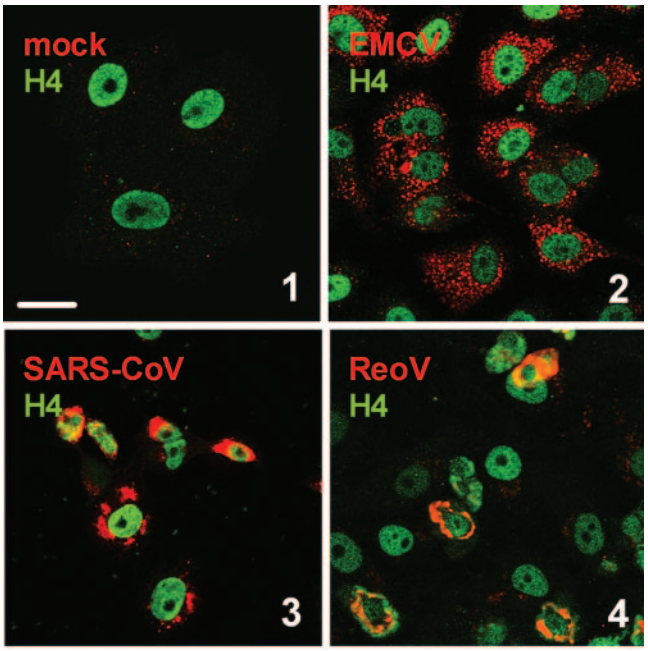

FIG. 2. dsRNA in cells infected with positive-strand RNA and dsRNA viruses. Vero cells were infected at a multiplicity of infection of 5 with EMCV (2), SARS-CoV (3), or ReoV (4) or left uninfected (1). At 5 h (EMCV), 16 h (SARS-CoV), or 48 h (ReoV) postinfection, cells were fixed and analyzed by immunofluorescence as indicated in the legend to Fig. 1. Bar, 20 μm. All pictures were taken with the same magnification. The differences in size and morphology of the nuclei are most probably caused by initiation of apoptosis or by disturbances in nuclear-cytoplasmic transport, as has been described for several viruses (6, 21, 23, 27).

clonal antibody J2 (Scicons, Hungary) was diluted 1:200 in PBS, and the fluorophore signal was visualized using the tyramide signal amplification (TSA) cyanine 3 system (Perkin plus Elmer). For viral immunofluorescence, cells were incubated with a rabbit polyclonal anti-FLUAV N (1:200) or a rabbit polyclonal rabbit anti-LACV N (1:500). For nuclear counterstain, the rabbit anti-acetyl histone H4 antiserum (Upstate) was used at a dilution of 1:500. After incubation at room temperature for 1 h, the coverslips were washed three times in PBS and then treated with the secondary antibody goat anti-mouse or anti-rabbit at a dilution of 1:200 each. Cells were again washed three times in PBS and mounted using Fluorsave solution (Calbiochem). Stained cell samples were examined using a Leica confocal laser scanning microscope with a 63× NA-1.4 objective. The confocal pinhole was set to 1 Airy unit, and pictures were digitally magnified twofold. The same microscope settings and exposure times were used within each set of experiments.

RESULTS

The monoclonal dsRNA-specific mouse antibody J2 specifically recognizes dsRNA of more than 40-bp length (52). It was previously used for an enzyme-linked immunosorbent assay and immunoblot analysis of virus-infected plants (37, 52) but, to our knowledge, not for immunohistochemistry of animal cells. We tested the suitability of J2 to detect transfected poly(I:C), a synthetic dsRNA, in Vero cells in situ. Figure 1 (panel 2) shows that dsRNA-transfected cells indeed give rise to a strong signal, whereas in nontransfected cells, a much weaker immunofluorescence is present (Fig. 1, panel 1). Importantly, the fluorescence signal was sensitive to the dsRNA-specific RNase III (Fig. 1, panel 3) but not to the single-stranded RNA (ssRNA)-specific RNase A (Fig. 1, panel 4) or to DNase I (data not shown), indicating that the target structure detected by J2 was authentic dsRNA. RNase III treatment also reduced the background fluorescence (data not shown), confirming previous findings of endogenous cellular dsRNA (32, 66).

Using the J2 antibody, we analyzed the dsRNA content of cells infected with positive-strand RNA viruses and dsRNA viruses. For members of the togaviruses (57) and the flaviviruses (33, 69), in situ detection of dsRNA has been performed previously. We therefore restricted our analysis to the remaining groups, namely, the picornaviruses represented by EMCV, the coronaviruses represented by SARS-CoV, and ReoV. As shown in Fig. 2, strong dsRNA signals were present after infection with all these viruses. Note that for this and all other studies, time points were chosen in which a peak signal for dsRNA was detected. Taking this together with data from the literature (33, 57, 69), we can conclude that infection with both positive-strand RNA and dsRNA viruses results in the production of significant amounts of dsRNA. This dsRNA is indeed of viral and not of cellular origin, since the dsRNA signal was also detected under treatment with the DNA-dependent RNA polymerase inhibitor actinomycin D (data not shown). Also, similar to the situation with synthetic dsRNA (Fig. 1), the viral dsRNA signal appears to consist of ssRNA hybrids, since RNase A treatment did not alter the immunofluorescence signal (data not shown).

Next, we monitored dsRNA synthesis in cells infected with DNA viruses. As shown in Fig. 3, all viruses tested, namely,

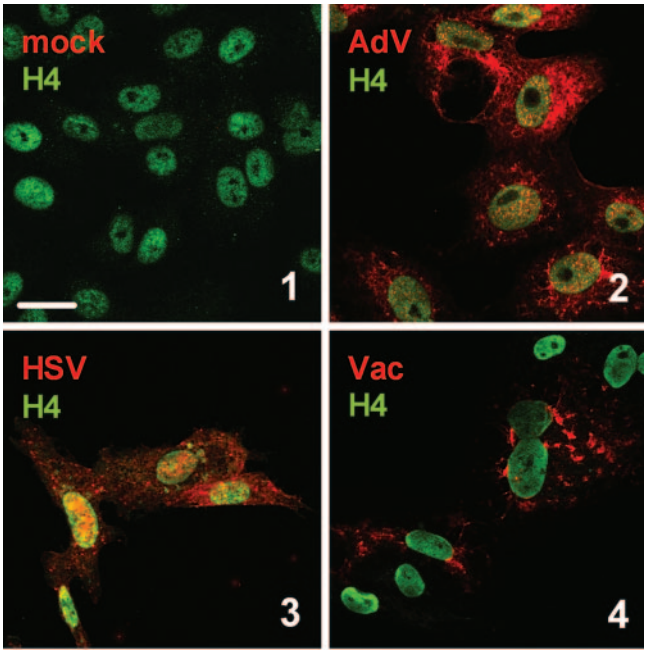

FIG. 3. dsRNA in cells infected with DNA viruses. Vero cells were infected at a multiplicity of infection of 5 with AdV (2), HSV (3), or Vac (4) or left uninfected (1). At 7 h (AdV, HSV) or 5 h (Vac) postinfection, cells were fixed and analyzed by immunofluorescence as indicated in the legend to Fig. 1. Bar, 20 μm. All pictures were taken with the same magnification.

AdV, HSV, and Vac, were invariably positive for dsRNA. Thus, although the nature of their genome is different from RNA viruses, DNA viruses apparently also produce dsRNA during their replication cycle.

The third large group of viruses comprises the negative-strand RNA viruses. As examples, we chose FLUAV, which replicates in the nucleus, and LACV, which replicates in the cytoplasm as most other RNA viruses do. Much to our surprise, when we investigated dsRNA in infected cells, we detected hardly any signals above background levels (Fig. 4A and B, panels 1 and 2). Occasionally, single cells exhibited a cytoplasmic dsRNA signal (curiously, also for the nucleus-borne FLUAV), but these were always exceptions. Nevertheless, the viruses replicated efficiently, as is demonstrated by the synthesis of viral nucleocapsid proteins (Fig. 4A and B, panels 3 and 4). To further strengthen this last point, we measured infectious particle production and viral RNA levels and did not find significant differences between positive-strand RNA viruses (exemplified by SARS-CoV) and negative-strand RNA viruses (exemplified by LACV) growing in Vero cells (data not shown). As with FLUAV and LACV, we were also unable to detect significant dsRNA signals for Sendai virus and Newcastle disease virus, two members of the *Paramyxoviridae* family with a strong ability to induce IFN (data not shown). Thus, despite having comparable efficiencies of growth and RNA synthesis, negative-strand RNA viruses appear to produce much less dsRNA than positive-strand RNA viruses and DNA viruses.

DISCUSSION

To our knowledge, no study was hitherto performed in which the production of intracellular dsRNA by different viruses was systematically investigated. The formation of dsRNA was thought to be a general feature of all viruses (25, 32), although direct in situ proof for this was provided only for a few viruses, notably ones with a positive-strand RNA genome

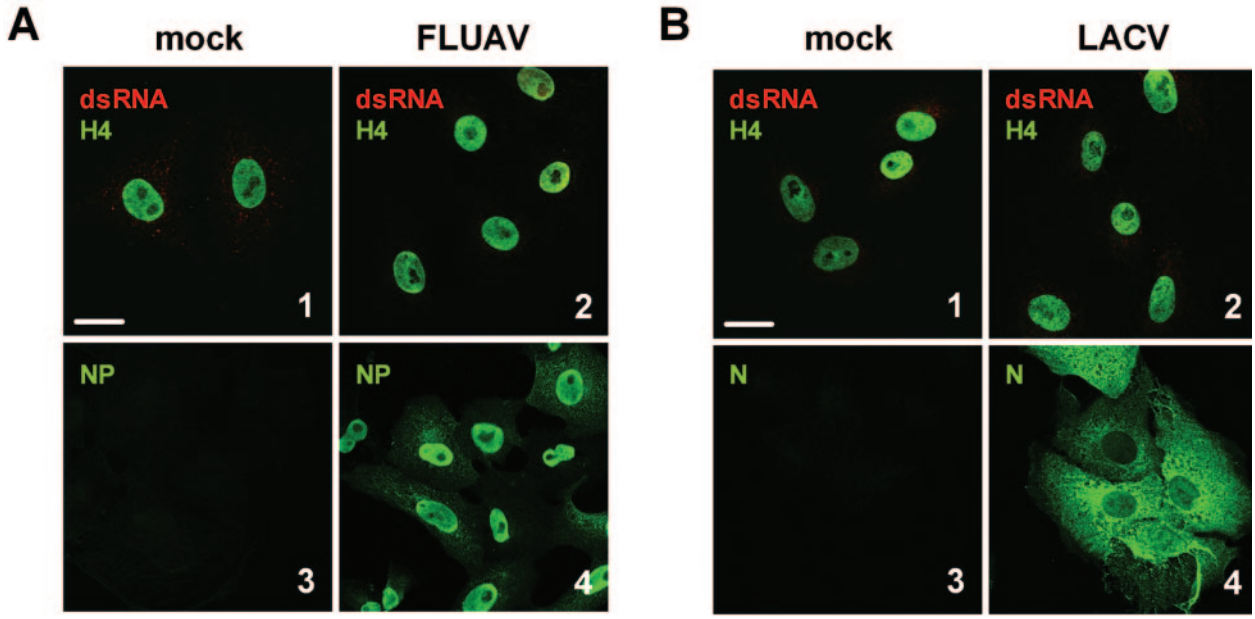

FIG. 4. Negative-strand RNA viruses. Vero cells were infected at a multiplicity of infection of 5 with FLUAV (A, panels 2 and 4) or LACV (B, panels 2 and 4) or left uninfected (A and B, panels 1 and 3). At 5 h postinfection, cells were fixed and analyzed by immunofluorescence either for dsRNA as indicated in the legend to Fig. 1 (A and B, panels 1 and 2) or for viral antigens (A and B, panels 3 and 4). Bar, 20 μm. All pictures were taken with the same magnification.

(33, 57, 69). Using a specific mouse monoclonal antibody, we were able to confirm the production of dsRNA by positive-strand RNA viruses, dsRNA viruses, and DNA viruses.

For negative-strand RNA viruses, however, we were unable to detect dsRNA in infected cells. In line with this finding, we have previously shown that expression of the dsRNA-binding domains of RIG-I or PKR in myeloid cells could abrogate the cytokine response only to the positive-strand RNA virus EMCV but not to the negative-strand RNA virus Sendai virus (45). This again indicates fundamental differences between different virus groups with respect to dsRNA formation. Nevertheless, it is well known that negative-strand RNA viruses can strongly induce IFN synthesis (18, 68), activate PKR or be sensitive to it (7, 56, 58), or express a dsRNA-binding protein, such as FLUAV NS1 (22, 36). However, since as little as one molecule of dsRNA per cell can be effective in triggering an antiviral response (42), it is possible that the amounts of dsRNA produced by negative-sense RNA viruses are below our detection limit. Alternatively, other viral structures may take the role of dsRNA as a danger signal for the host cell. Indeed, it was previously shown that the ribonucleoprotein particles (RNPs) of the negative-strand vesicular stomatitis virus (VSV) and measles virus are capable of triggering IFN induction (62, 63). In addition, ssRNAs of FLUAV and VSV are capable of triggering IFN induction via TLR7 (13, 38), and the FLUAV NS1 protein also binds to viral ssRNA (22). Moreover, PKR can also be activated independently of dsRNA by cellular proteins, such as PACT/RAX (24, 47). Since these proteins are stress activated, it is conceivable that PKR activation in response to negative-strand RNA virus infections occurs mostly via PACT/RAX and not via dsRNA. Also, the importance of dsRNA binding for the anti-IFN activity of FLUAV NS1 is still a matter of debate (19, 30). Indeed, the dsRNA-binding activity is not absolutely required for the inhibition of IFN induction (14), and NS1 confers a host of other dsRNA-independent activities (15, 30). Interestingly, to our knowledge, FLUAV NS1 is the only anti-IFN protein of a negative-strand RNA virus known to directly bind dsRNA. All other members of this taxonomic group rely on inhibiting other downstream parts of the IFN induction signaling chain, such as MDA-5 (4), the IRF-3 kinase TBK-1 (53, 65), IRF-3 (5, 11, 12, 26, 29), the RNA polymerase II complex (9, 10, 17, 34, 64), nuclear export of RNAs (16), and translation (1). On the other hand, however, the dsRNA-binding proteins MDA-5 (which is inhibited by paramyxoviruses) (4) and RIG-I both are important sensor molecules for negative-strand RNA viruses (45, 48, 71). Also, expression of a dsRNA-binding protein can rescue VSV from the antiviral effects of IFN (54). Thus, we cannot exclude the presence of small amounts of dsRNA produced by negative-strand RNA viruses, but we suppose that in the case of this virus group, other signals, such as ssRNA and RNPs, may be equally important for the host as danger signals in triggering an antiviral response. This view is not contradicted by previous reports about biologically active dsRNA derived from FLUAV-infected cells (41), since those studies involved the extraction of RNA from infected tissue and the removal of all proteins, thus allowing hybridization of RNAs which may have been well separated from each other before disruption (25).

It is tempting to speculate that negative-strand RNA viruses avoid the formation of dsRNA by packaging the genomic and antigenomic RNAs into RNPs. For most other viruses, hiding or sequestering of once-formed dsRNA is the strategy for circumventing activation of the innate immune system (68). The dsRNA of ReoV, for example, remains within the inner capsid throughout the viral replication cycle (25), and positive-strand RNA viruses replicate their genome enclosed in membrane vesicles (49). Many viruses, e.g., poxviruses, also express dsRNA-binding proteins (25). One may therefore wonder why dsRNA can nevertheless be detected in cells infected with ReoV (Fig. 2, panel 4) or Vac (Fig. 3, panel 4). Most likely, either the monoclonal antibody J2 can access dsRNA even when it is bound by a protein or the amount of dsRNA can exceed that of the virus-expressed proteins. Also, it was suspected that ReoV subviral particles only imperfectly cover the dsRNA (25). This view of leaky anti-dsRNA mechanisms is supported by the fact that ReoV can partly activate PKR (35) and that Vac needs to express the PKR decoy substrate K3L in addition to the dsRNA-binding E3L to overcome IFN sensitivity (25).

The origin of the dsRNA structures detected for positive-strand RNA viruses and for DNA viruses remains to be determined. For positive-strand RNA viruses, it could be either hybrids of cRNA strands generated during genome replication and transcription or intramolecular secondary structures within ssRNA molecules. Whereas the evidence for RNA-RNA hybrids in infected cells remains problematic due to the above-mentioned annealing artifacts during RNA extraction (25, 40), the latter view is supported by studies showing that highly structured viral ssRNAs are sufficient to trigger activation of PKR (44), 2-5-OAS (39), or RIG-I (59). For DNA viruses, dsRNA may arise as a result of overlapping converging transcription (25, 40) or highly structured ssRNAs, such as the adenovirus virus-associated RNAs, of which more than 10^8 copies are present in a single cell (43). The fact that the ssRNA-specific RNase A has no effect on the viral dsRNA signal does not absolutely rule out the presence of intramolecular secondary structures. Rather, it is still possible that sterical constraints do not allow the enzyme to access the single-strand regions and cut off the secondary structures.

In summary, we have demonstrated by a nondestructive in situ analysis that production of dsRNA occurs for positive-strand RNA viruses, dsRNA viruses, and DNA viruses. Negative-strand RNA viruses, however, appear to represent an exception to that rule since no significant dsRNA signals were detected. Most probably, other molecular patterns, such as RNPs and ssRNA, are more dominant as danger signals for the immune system.

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