The Poly(C) Region Affects Progression of Encephalomyocarditis Virus Infection in Langerhans' Islets but Not in the Myocardium

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The diabetogenic variant PV2 of encephalomyocarditis virus was cloned, and three recombinants differing in their 5' poly(C) tracts were analyzed. It is shown that the poly(C) region is not essential for infectivity in mice but does influence the virus load and degree of pathological lesions within the Langerhans' islets but not in the myocardium.

Variant PV2 of encephalomyocarditis virus (EMCV) induces an insulin-dependent diabetes mellitus-like syndrome in certain inbred mouse strains by destruction of insulin-producing pancreatic beta cells (14, 17). To analyze further the molecular basis of the pathogenic properties, PV2 was cloned and sequenced (17).

A feature of murine cardioviruses (EMCV and mengovirus) and bovine aphthoviruses (FMDV) is the homopolymeric cytosine tract (60 to 350 C's) within the 5' noncoding region. The length of this region and the distance from the 5' end of the RNA depends on virus strain and isolate (3, 4). The task of the poly(C) region in the viral replication cycle is not yet clear; however, it is not likely that it plays a direct role in viral RNA replication or protein synthesis, since cardioviruses with shortened or deleted poly(C) tracts replicate efficiently in cell culture (2, 7, 10, 15). On the other hand, at least in the case of mengovirus the length of the poly(C) tract seems to influence viral pathogenesis upon inoculation into mice, i.e., the fewer the C's, the more attenuated appears the corresponding virus (6, 15, 18). In contrast to mengoviruses, a full-length poly(C) region seems not to be a prerequisite for infectivity of EMCV applied intracerebrally (10). To further elucidate the function of the poly(C) region for the EMCV life cycle, construction of cDNA clones with modified poly(C) tracts was intended. Since long dC · dG tracts are neither strictly reproduced from template-dependent polymerases nor precisely maintained in the bacterial host, several approaches have been used to overcome this problem (1, 7, 16, 19, 22). To begin with, we constructed and sequenced a full-length cDNA clone of PV2 from overlapping fragments (17). Although the original poly(C) region of EMCV PV2 is composed of 117 or 118 cytosines, after reverse transcription and further cloning procedures the longest poly(C) tract amounted to only 20 cytosines (rPV2/C20 in Fig. 1A). To elongate the 5' noncoding region, the *EcoRV*-*HindIII* fragment of the clone rPV2/C20 was replaced by the corresponding fragments of pSPORT/EMC-ran or pSPORT-

EMC-dT, leading to rPV2/ran and rPV2/dT, respectively (Fig. 1). The former two clones were created by introducing a "random" sequence or, alternatively, a poly(T) region into pSPORT/EMC5'-PCR1+2 as described previously (22). Clone pSPORT/EMC5'-PCR1+2 is derived from highly virulent EMCV variant PV21 and maintains a poly(C) region of about 20 cytosines, including an *SmaI*-*SalI* cloning site in the approximate center of that tract.

The "ran" sequence is of unknown origin (ran is derived from random) and was introduced into the *SmaI* site of pSPORT/EMC5'-PCR1+2 during the attempt to clone polydeoxycytidyl · polydeoxyguanyl (Boehringer Mannheim) as described previously (22). After analysis of the resulting clones by restriction enzymes, it was found that the longest insert was not, as expected, composed of poly(C) but of a "normal" sequence (Fig. 2A). The poly(T) region was synthesized by the terminal deoxynucleotidyl transferase (TdT). In contrast to the results for poly(C) tracts, by use of TdT a poly(T) region of about 100 nucleotides can be created and poly(T) regions are correctly transmitted through bacterial hosts.

Because of this cloning procedure, rPV2/C20 on the one hand and rPV2/ran and rPV2/dT on the other hand differ not only in the poly(C) region and its immediate vicinity but also by virtue of 13 single-base exchanges and 4 single-base insertions derived from sequence deviations between PV2 and PV21 (Fig. 2B).

In vitro-transcribed RNAs of all clones were shown to be infectious in transfection experiments, and active recombinant virus could be isolated from the supernatant. There are no significant differences observed in the growth characteristics of the three recombinants in cell culture. The strains form plaques of approximately the same size within a defined time interval, and endpoint dilution experiments reveal similar infectivities of in vitro-transcribed RNA (data not shown). The infectivities of the RNA of the variants are about 10 times lower than that of the original PV2 RNA (data not shown). The interferon (IFN) levels of uninfected and infected mice have not been determined. For the EMCV variants D and B (D, diabetogenic; B, nondiabetogenic), only the nondiabetogenic variant B is a good inducer for IFN; therefore, it is suggested that IFN plays a major role in protecting the pancreatic beta cells from destruction (5, 12, 20, 21). It is reported that this effect depends, e.g., on the mouse strain (8, 13) or the "interferon-inducing particle" phenotype of the virus (11). However, it could be shown that a non-IFN-inducing subvari-

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FIG. 1. Schematic drawing of the construction of the three recombinant clones rPV2/C20, rPV2dT, and rPV2/ran. (A) Viral RNA of the EMCV variant PV2 was cloned and sequenced (17). In order to minimize the distance between the T7 promoter and the first nucleotide of the viral sequence, the last cloning step was performed in pT7T3 (GIBCO BRL). After *EcoRI* digestion and S1 nuclease treatment only an additional C residue was left in front of the viral 5' end. *EcoRI* cleaves the vector just in front of the T7 promoter, and the recognition site of this enzyme was added to the viral 5' end by an elongated primer as described earlier (22). (B) The construction of pSPORT/EMC5'-PCR1+2 has been described earlier (22). Efforts to clone poly(dC) · poly(dG) fragments were not successful but led to the cloning of a random sequence (ran). Elongation of the 3' end generated by *SmaI* digest of pSPORT/EMC5'-PCR1+2 with the terminal deoxynucleotidyl transferase gave rise to a poly(T) region of at least 90 thymines. The position of the *HindIII* site depends on the length of the poly(C) region or its equivalent. The original poly(C) region of PV2 amounts to 118 nucleotides (nt). C20 is composed of 20 nt, ran is composed of 238 nt, and dT is composed of 115 nt.

FIG. 2. (A) Sequence of the ran insertion including the flanking 10 and 14 Cs of plasmid rPV2/ran. (B) Sequence alignment of the *EcoRV*-*HindIII* fragments (positions 44 to 470 in the PV2 sequence) of the recombinant clones rPV2/C20, ran, and dT (see text). Single-base exchanges and single-base insertions resulting from sequence variance between PV2 and PV21 are indicated. Deletions are marked by dashes, and identical bases are marked by points.

ant of EMCV-B also protects mice against the production of diabetes by EMCV-D (9). Hence, the significance of IFN in various experimental models is not easy to ascertain.

In order to investigate the pathogenicity of these viruses, juvenile (4-week-old) NMRI mice were inoculated intraperitoneally with 5×10^5 PFU of one of the recombinant viruses or the original PV2 and were weighed daily. The full-length clone of the EMCV variant PV21 was not used as a control since the pathognomic lesions induced by this variant differ dramatically from those induced by PV2; e.g., the exocrine and not the endocrine pancreatic tissue is destroyed and PV21 causes a generalized infection with 100% lethality in adult mice, whereas PV2 is only lethal for mice up to 4 or 5 weeks old. Hence, the validity of such a control is questionable. In par-

ticular, our full-length clone of PV21 containing the poly(U) region does not replicate in mice.

Regardless of the structure of the viral 5' region, the recombinant viruses were able to replicate in animals since all mice inoculated lost weight (Fig. 3), a typical sign of productive viral infection (unpublished data). Following inoculation with rPV2/dT, 100% lethality was seen within the first 6 days postinoculation (p.i.) as compared to 100% lethality with PV2 within the first 4 days p.i. (Fig. 3). After infection with rPV2/C20 four of five mice died within the first 7 days p.i.; after infection with rPV2/ran, five of six mice died within the same period. The surviving two mice recovered and reached the weight of uninfected controls within a few days.

To determine the extent of replication of the virus in various organs within the first 3 days after virus inoculation, the 50%

FIG. 3. Relative mouse weights of 4-week-old NMRI mice infected with PV2 or one of the recombinant derivatives in percent (weight on day 0 = 100%). Mouse inoculations were as follows: PV2, five mice; rPV2/C20, five mice; rPV2/dT, four mice; rPV2/ran, six mice; rPV2/dT, four mice. None of the PV2-infected mice survived day 3 p.i., and none of the rPV2/dT-infected mice survived day 5. For the two other recombinant viruses, in each case one mouse recovered and survived.

FIG. 4. Mean $TCID_{50}$ s of heart, pancreas, and brain for juvenile (4-week-old) NMRI (A) and adult (12-week-old) SJL mice (B). In each case four animals were inoculated with 5×10^5 PFU of either PV2 (solid rhomb), rPV2/C20 (circle), rPV2/ran (square), or rPV2/dT (triangle) and sacrificed at the indicated days p.i. Organs were prepared, and the $TCID_{50}$ s were determined. Due to the high levels of lethality of the viruses tested, the $TCID_{50}$ s of the organs of juvenile mice could be determined only until day 3 p.i.

FIG. 5. Histological examination and in situ hybridization of heart (A and B) and pancreas (C and D) sections of adult mice infected with rPV2/ran or rPV2/dT. Magnification, $\times 400$. (A) Typical histologic abnormalities found on day 7 p.i. in the myocardia of SJL mice infected with 10^5 PFU of rPV2/ran. A focus of infection is established after extensive resorption of necrotic myocytes by the macrophages. The infiltrate contains only a few lymphocytes (tight nuclei). Comparable signs are found after infection with the two other recombinants or wild-type PV2. (B) Myocardial section of an rPV2/ran-infected mouse (day 7 p.i.) after hybridization to a ^{35}S -labelled probe representing the sequence of the first 157 amino acids of capsid protein VP3. Autoradiographic signals are localized over cardiomyocytes. (C) Two neighboring Langerhans' islets (lighter stained area) of an rPV2/dT-infected animal on day 4 p.i. Extensive necrosis of islet cells, surrounded and superimposed by dense aggregates of lymphocytes and macrophages, is visible. The infiltrates resorb the affected islet cells. (D) Another islet of the same animal described in panel C after in situ hybridization. Pronounced signals can be recognized over approximately 20% of the islet tissue. Slight degenerative alterations are closely associated with isolated pleomorphic mononuclear cells.

tissue culture infectious doses (TCID_{50}s) of recombinant virus isolated from the main target organs of EMCV, i.e., heart, brain, and pancreas, were determined. For this purpose, 4-week-old NMRI mice were inoculated as described above and sacrificed 24, 48, and 72 h p.i. The organs from four mice per virus variant and time point were prepared, and following homogenization TCID_{50}s were determined by the endpoint dilution method. Despite the apparently decreased pathogenicity of the recombinant variants as compared with that of PV2 (Fig. 3), no considerable differences in the final infectivity

loads were ascertained (Fig. 4A). However, a tendency for more rapid and slightly increased virus replication of PV2 as well as of rPV2/dT was apparent.

To analyze the infectivities of the recombinant clones in immunocompetent adult animals, 12-week-old SJL mice were inoculated intraperitoneally as described previously (17), and on days 1, 2, 3, 5, and 7 p.i., mice were sacrificed and organs were prepared. In addition, on days 5 and 7 p.i. blood glucose values were determined prior to sacrificing. SJL mice are, in contrast to NMRI mice, susceptible to diabetes induction by EMCV.

For all variants, histological examination of heart sections revealed multiple foci of myocardial cell necrosis with subsequent macrophage infiltration (Fig. 5A). Virus loads and primary virus replication were determined by organ titration and in situ hybridization. In the hearts of adult mice, the final TCID₅₀s produced by PV2 were nearly reached by the recombinants—though they were significantly delayed by 2 to 3 days (Fig. 4B). In situ hybridization experiments, too, revealed similar loads of viral RNA in the myocytes of infected SJL mice independent of the variant (Fig. 5B). In summary, as far as infection of the heart is concerned, no significant difference between the viruses tested could be observed.

In contrast, remarkable differences in the degree of pathological lesions in the pancreas were measurable. Insulinitis and frank necrosis of islet cells followed by a resorptive infiltrate composed of macrophages as well as of granulocytes and some lymphocytes, as described for PV2 infection (17), are only observed after infection with rPV2/dT (Fig. 5C). Also, strong signals in the islets of rPV2/dT-infected mice were detected by in situ hybridization experiments (Fig. 5D). Nevertheless, the islet damage does not match the extent of PV2-induced lesions described earlier (17) and appears insufficient to cause a diabetes syndrome, as blood glucose values of mice infected with rPV2/dT were not increased.

In PV2/C20-infected mice just slight histomorphological changes of the islets of Langerhans, such as eosinophilic necrosis or nuclear condensation, could be observed, and in in situ hybridization experiments only weak signals of viral RNA could be detected in some islets of Langerhans (data not shown). Nevertheless, with a delay of one day this variant multiplied to approximately the same titer as the original PV2 (Fig. 4B). This indicates that the degree of tissue damage must not necessarily be reflected by the total virus load of the organ.

Neither cell damage nor positive signals after in situ hybridization are noticed in the Langerhans' islets of rPV2/ran-infected mice. Furthermore, only weak replication is observed in the pancreas, since the TCID₅₀s declined from day 2 on, and, as after infection with rPV2/dT, on day 7 p.i. no infectious virus was detectable. Both rPV2/C20 and rPV2/ran were incapable of inducing an elevated blood glucose level.

The infectivity titers of recombinant viruses in the brains of adult SJL mice are remarkably lower than those obtained after PV2 infection. Accordingly, we suppose that successful infection of the brain might also depend on an intact poly(C) tract.

Conclusions. Although the poly(C) region apparently is dispensable for virus replication in mice, this stretch appears not to be a simple "distance-keeper" which can be replaced, e.g., by a poly(T) region. Whereas severe myocarditis, most often within the inner third of the left ventricle, was found after infection with each of the three recombinant viruses, significant insulinitis was only observed after infection with rPV2/dT. Nevertheless, the diabetogenic character of PV2 was not matched by that of rPV2/dT, presumably since sufficient islets stayed unaffected by rPV2/dT infection. Both variants with a pyrimidine stretch (rPV2/C20 and rPV2/dT), however, replicate slightly more efficiently than rPV2/ran, which contains a nonviral normal sequence instead of the poly(C) region. Hence, a long pyrimidine stretch, i.e., of at least more than about 20 nt, in the 5' region of the viral genome seems to be a prerequisite for successful infection of the Langerhans' islets, whereas this region does not influence viral replication in the myocardium. As already proposed for mengovirus (6, 18), the

influence of the homopolymeric cytidine stretch on viral pathogenesis appears to be target tissue dependent.

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