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Isolation and Characterization of an Endogenous Type C Virus of Rhesus Monkeys

Abstract. *A type C retrovirus was isolated from a continuous cell line established from a spontaneous esophageal carcinoma of a rhesus monkey (*Macaca mulata*) by prolonged cocultivation with canine cells. A DNA transcript of the viral RNA hybridized to a high level and kinetic analysis indicated the presence of multiple copies of the viral genome in rhesus monkey DNA, showing that the virus is endogenous in this species. The rhesus monkey virus closely resembles, in several respects, an endogenous type C virus previously isolated from stumptailed macaques* (Macaca arctoides), *a species closely related to rhesus monkeys.*

We previously reported the establishment of an epithelial cell line, 816A, from a lymph node metastasis of a spontaneous, esophageal carcinoma of a rhesus monkey, *Macaca mulata*. At early passage levels, 816A cells showed both budding and extracellular type C virus by electron microscopy (*1*). In an effort to isolate this virus, 816A cells were cocultivated with cell lines of human (A204, HOS), mink (CCL64), bat (B88), and dog (Cf2Th, D-17) origins after treatment with 5-iodo-2′-deoxyuridine (30 μg/ml) and dexamethasone (0.1 μg/ml). Mixed cell cultures were passaged twice monthly, and clarified, 600-fold concentrated culture fluids were tested weekly for reverse transcriptase (RT) activity with the template poly(rA)·oligo dT_{12-18}, where rA is riboadenylate and dT is deoxythymidylate.

Reverse transcriptase assays for the first 5½ months after initiation of cocultivation were negative for all 816A cell combinations. In the sixth month, at post-cocultivation passage 15, the 816A/D-17 culture was positive for RT activity at a low level, 40 pmole per milliliter per hour above a background of 3.2 pmole per milliliter per hour. Similar weak RT activity was found over the next several passages. Electron microscopy of 816A/D-17 cultures at passages 16 and 17 revealed low concentrations of mature type C virus. At passage 17, 500-fold concentrated culture fluids from 816A/D-17 cultures were inoculated onto Polybrene-treated Cf2Th, CCL64, and A204 cells. At post-inoculation passage 13, 100-fold concentrated culture fluids of Cf2Th cells were positive for RT activity. Such activity increased in culture fluids over the next several passages to levels of 480 pmole per milliliter per hour above background. The RT activity showed preferences for the synthetic template poly(rA)·oligo dT_{12-18} compared to poly(dA)·oligo dT_{12-18} and for Mn^{2+} over Mg^{2+}, indicating type C virus activity. The inoculated A204 and CCL64 cells were negative for RT activity through passages 12 and 13, respectively. It was possible to develop positive RT activity in an additional 816A/D-17 cocultivation experiment over essentially the same time and passage levels after cocultivation. Prior to and during the entire period over which the virus was isolated from the 816A cells, no MAC-1 virus, the endogenous virus of stumptailed macaques (*Macaca arctoides*) (*2*), was present in our laboratory. We have designated the rhesus monkey cell isolate as MMC-1 (*Macaca mulata* type C, first isolate). For initial characterization MMC-1 was grown in Cf2Th cells.

In addition to having an RT characteristic of type C viruses, electron microscopic examination of MMC-1 revealed early, intermediate, and late budding stages and immature and mature extracellular virions typical of type C viruses. The mature, extracellular virus has a diameter of 110 nm and the nucleoid mea-

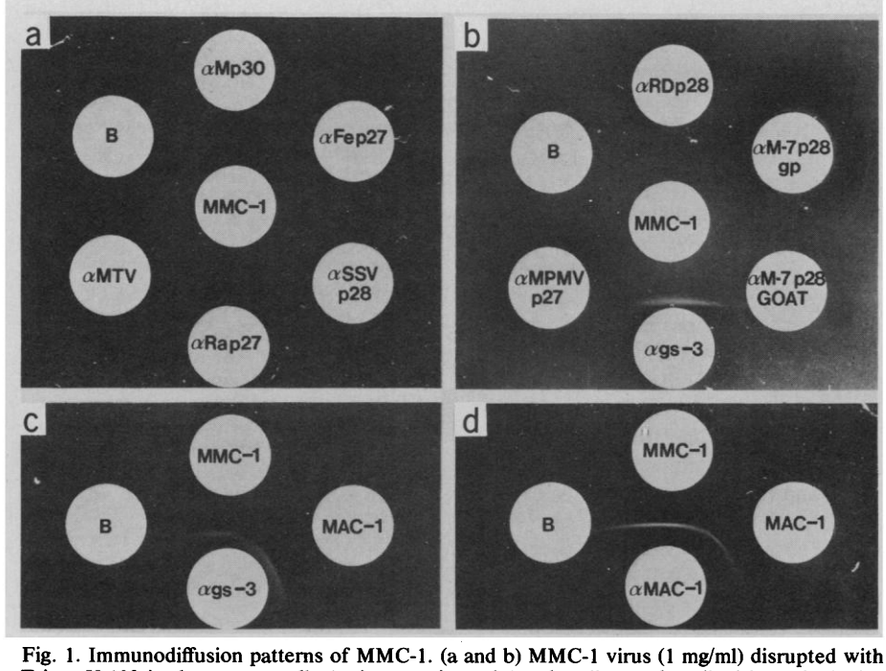

Fig. 1. Immunodiffusion patterns of MMC-1. (a and b) MMC-1 virus (1 mg/ml) disrupted with Triton X-100 in the center well. Antiserums in peripheral wells are described in a clockwise direction from the top well. (a) All antiserums of caprine origin. Antiserum to Rauscher murine leukemia virus p30 (*αMp30*), antiserum to feline leukemia virus p27 (*αFep27*), antiserum to simian sarcoma virus p28 (*αSSVp28*), antiserum to rat type C virus p27 (*αRap27*), antiserum to mouse mammary tumor virus (*αMTV*), buffer (B). (b) Caprine antiserum to RD-114 virus p28 (*αRDp28*), guinea pig antiserum to endogenous baboon virus p28 (*αM-7p28 gp*), caprine antiserum to M-7p28 (*αM-7p28 goat*), interspecies caprine antiserum to mammalian type C virus p30s (*αgs-3*), caprine antiserum to Mason-Pfizer monkey virus p27 (*αMPMVp27*), buffer (*B*). (c and d) MMC-1 virus and MAC-1 virus both at 1 mg/ml and both disrupted with Triton X-100. (d) In the lower well, caprine antiserum to purified MAC-1 virus [see (*4*)].

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sures 75 nm in diameter. In sucrose gradients, MMC-1 has a buoyant density of 1.14 g/cm^3.

The MMC-1 was tested with antiserums reactive with the major structural protein of various mammalian retroviruses by immunodiffusion in agar. Figure 1, a and b, shows that there was no reaction with any of these antiserums with the exception of a broadly reactive caprine serum, designated αgs-3 (where α represents anti), that has antibody mainly to the p30 determinants shared by the known mammalian type C viruses (*3*). Figure 1c shows the reaction of MMC-1 with this serum in comparison with MAC-1. The line of identity indicates a close antigenic relationship between these two viruses. This close relationship was further illustrated when MMC-1 and MAC-1 were tested with an antiserum to MAC-1 (Fig. 1d) (*4*). The structural proteins of MMC-1 were compared with those of other retroviruses by SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis. This comparison showed that the protein profiles of MMC-1 and MAC-1 are very similar, having major proteins of apparent molecular weights of, respectively, 73,000 and 70,000 and 26,000, 12,000, and 10,000.

To determine if MMC-1 is an endogenous virus of rhesus monkeys, we prepared a complementary [^{3}H]thymidine-labeled DNA transcript, [^{3}H]cDNA, of the viral RNA. This [^{3}H]cDNA hybridized to RNA from MMC-1 at a level of 84 percent, to RNA from MAC-1 at a level of 80 percent, and showed no hybridization to itself. Table 1 shows the results of hybridization assays with the MMC-1 [^{3}H]cDNA and various DNA preparations. A high level of hybridization was found with DNA prepared from normal rhesus monkey liver and spleen, whereas human, normal canine, and normal murine DNA were all essentially negative. The cell culture line in which the virus was initially detected by cocultivation, D-17, and the line in which it was propagated for study, Cf2Th, were also negative. The viral [^{3}H]cDNA reassociated with rhesus monkey DNA with a $C_0t_{1/2}$ (where C_0t is moles of nucleotide per liter times seconds) of approximately 15. As nonrepeated cell DNA reassociates under these conditions with a $C_0t_{1/2}$ of 3300, we estimate that there are about 200 viral copies per haploid cell genome.

MMC-1 is the first endogenous retrovirus isolated from rhesus monkeys and the second type C virus isolated from macaques. The isolation of MAC-1, the first endogenous type C virus recovered from macaques, required protracted cocultivation with cells of a heterologous species as did the isolation of MMC-1. On the basis of data of Todaro and coworkers (*2*) showing that an MAC-1 cDNA probe hybridized to rhesus monkey DNA at a level of 96 percent, one would predict that an endogenous type C virus of the rhesus monkey would be closely related to MAC-1. The immunological findings relative to the major core protein, the molecular weights of the viral structural proteins, the relatively low buoyant density, the similarly high copy number in cells of the host species, and the high level of hybridization of MMC-1 [^{3}H]cDNA to MAC-1 RNA support this prediction. Thus, the endogenous macaque viruses may be as closely related to each other as are the endogenous type C viruses of different species of baboons: *Papio cynocephalus*, *P. hamadryas*, *P. papio*, and the gelada, *Theropithecus gelada*. Distinctions can be made among these viruses by immunological analysis of their lower molecular weight structural proteins and by molecular hybridization techniques (*5*), even though their major structural proteins are antigenically identical and levels of cross-hybridization are high. The infrequency of virus isolation from macaque cells suggests that these cells are capable of effectively suppressing their high levels of endogenous viral genomes. Such suppression was at least temporarily lost in the case of early-passage 816A cells, where low levels of complete virus were expressed.

Rhesus monkeys are the most commonly used primates for general research purposes and they are extensively used for the production and testing of biologics (*6*). They have also been used successfully in chemical carcinogenesis programs (*7*). It will now be important to assess the role of MMC-1 in these areas, especially in terms of its possible relationship to tumor induction and its presence in biologics.

Table 1. Molecular hybridization of MMC-1 [^{3}H]cDNA. The MMC-1 [^{3}H]cDNA was synthesized in a 250-μl reaction mixture containing 1 m*M* deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate, 0.15 m*M* ^{3}H-labeled deoxythymidine triphosphate (44 Ci/mmole), 0.04 percent NP-40 (a detergent), 0.05*M* tris-HCl (*p*H 8.3), 5 m*M* magnesium acetate, 0.05*M* NaCl, 0.01*M* dithiothreitol, actinomycin D (100 μg/ml), and about 2.3 mg of virus. After 2 hours, SDS was added to 1 percent and the mixture was centrifuged at 40,000 rev/min for 2½ hours at 10°C in an SW-41 rotor through a 15 to 30 percent sucrose gradient. A sharp peak of trichloroacetic acid insoluble radioactivity was observed in the middle of the gradient. The pooled fractions were incubated in 0.3*N* NaOH for 3 hours at 37°C, neutralized, and extracted with phenol followed by chloroform. The [^{3}H]cDNA was collected by ethanol precipitation. Viral RNA was prepared as described by Schlom *et al.* (*8*) and DNA was prepared according to the urea-phosphate method (*8*). Hybridizations were performed with 1 to 3 × 10^3 count/min of [^{3}H]cDNA and 100 to 500 μg of DNA in 1*M* sodium phosphate buffer, *p*H 6.8, at 65°C. After appropriate times the samples were diluted to 0.14*M* phosphate buffer and 0.01 percent SDS, applied to hydroxylapatite columns at 50°C, and eluted at 10°C increments. Fractions were assayed for radioactivity after addition of 2 volumes of Aquasol II. The extent of hybridization is defined as the percentage of total radioactivity eluting from the column above 50°C, and C_0t values are corrected to the standard conditions of 0.12*M* phosphate buffer (*8*). In all positive samples, >90 percent of the hybridized [^{3}H]cDNA eluted above 70°C, indicating well-matched duplexes.

DNA preparation	C_0t	Percentage hybridized
Rhesus monkey liver	12,000	72
Rhesus monkey spleen	3,100	71
MMC-1-infected D-17 cell	5,700	70
MMC-1-infected Cf2Th cell	5,700	65
Uninfected D-17 cell	3,200	2
Uninfected Cf2Th cell	3,500	2
Human (A204 cell)	6,200	2
Canine liver	5,400	3
Murine liver	8,600	6
None		<1

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