Type C oncornavirus isolation studies in systemic lupus erythematosus

II. Attempted detection by viral RNA-dependent DNA polymerase assay

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SUMMARY Isolation of type C oncornavirus was attempted from 20 tissues and cell cultures of patients with systemic lupus erythematosus. Chemical inducers, cocultivation and fusion with cells from multiple other species, prolonged subculturing, and the RNA-dependent DNA polymerase assay for virus detection were used. A type C virus was isolated, but was shown to be the endogenous rat virus. Thus the methods, although generally appropriate, were not specifically permissive for replication of a human type C virus. This agrees with the failure of other investigators to isolate a virus of undisputed human origin. Combining available evidence, a fundamental role for type C viruses in lupus erythematosus remains an attractive hypothesis.

It has been postulated that viruses are involved in the pathogenesis of systemic lupus erythematosus (SLE) (reviewed in Phillips and Christian, 1978). Particular interest has been shown in type C oncornaviruses because of their role in the animal model of SLE, New Zealand (NZ) mouse disease (Yoshiki *et al.*, 1974; Levy *et al.*, 1975). However, the evidence is conflicting as to whether type C expression is enhanced, similar, or absent in SLE patients compared with other humans (Markenson and Phillips, 1978). Our earlier study failed to isolate type C, or other RNA viruses, or mycoplasma from SLE cell cultures and tissues (Phillips *et al.*, 1976). In this study we used more sensitive methods previously successful in isolating type C viruses from other species, and the RNA-dependent DNA polymerase (RDDP) assay for virus detection.

Materials and methods

PATIENTS

We studied 13 patients with SLE (Cases 1–13, Table 1) according to the American Rheumatism Association criteria (Cohen *et al.*, 1971) and 6 with probable or possible SLE (Cases 14–19). All were female, aged 15–65 years (mean 33.0 years), and had

been ill for 0.5–15 years (mean 6.7 years). 7 had clinically active disease and 12 were receiving prednisone, 1–60 mg daily (mean 27.0 mg); 5 were receiving immunosuppressive drugs. 20 tissues (Table 1) were studied. Cases 1–5 had also been tested previously (Cases 2, 3, 5, 6, 7 respectively in Phillips *et al.*, 1976).

CELL CULTURES

SLE tissues were obtained and the cells cultured as described (Phillips *et al.*, 1976) except for tissues from Cases 8 and 17, which were frozen at -70°C until testing. 31 experiments were done: 5 with frozen tissue homogenates, the remainder with SLE cells periodically subcultured for up to 18 months before testing (6 tested immediately or cultured for <1 month, 8 cultured for 1–6 months, 7 for 6–12 months, and 5 for 12–18 months; Table 1).

For virus isolation, 6 cell lines from heterologous species were used: rabbit cornea (SIRC; CCL 60, American Type Culture Collection, Rockville, MD), mink lung (Mv 1 Lu; CCL 64), bat lung (Tb 1 Lu; CCL 88), horse skin (E. Derm.; CCL 57), fetal rhesus monkey lung (DBS-FRHL-1; from Dr J. C. Petricciani, Food and Drug Administration, Rockville, MD), and Harvey murine sarcoma virus-transformed nonvirus-producing rat kidney (H-NRK; from Dr T. Pincus, Memorial-Sloan

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Table 1 Type C virus isolation from SLE tissues and cell cultures: specimens and methods used

Case no.	Tissue	No. of experiments	SLE cell culture before testing		Test method†	No. of different heterologous cell lines used	No. of subcultures done/cell line	
			Duration (m)	Induction*			Mean	Range
1	Spleen	2	13, 17	I, ID	RC, RF	6	6.8	3-8
2	Synovium	2	13, 18	I, I	C, C	7	7.3	2-9
3	PC‡	2	8, 11	I, I	RC, RC	5	3.7	1-9
4	Spleen	2	6, 8	I, I	RC, RC	6	6.6	1-11
5	Placenta	2	2, 9	I, ID	C, RF	8	4.4	1-6
6	PC	1	0	I	C	6	8.0	8
7	Thymus	2	4, 9	I, ID	RC, RF	8	6.5	1-8
8A	Kidney	1	—	—	Inoc	6	7.2	4-8
8B	Spleen	2	—	—	Inoc (2)	7	10.8	3-16
9	PC	1	1	ID	F	10	2.8	1-9
10	Skin-muscle	1	14	ID	RF	4	3.8	1-6
11	Synovium	1	4	IDT	F	9	8.1	3-9
12	PC	1	2	IDT	F	9	12.0	12
13	Placenta	1	0	—	F	8	11.5	6-13
14	PC	1	1	I	C	7	7.1	1-9
15	Placenta	1	1	ID	RC	9	8.6	4-11
16	Kidney	3	2, 5, 9	I, I, ID	C, C, RF	9	2.8	1-6
17	Spleen	2	—	—	Inoc (2)	6	6.5	1-11
18	Spleen	2	0, 4	I, I	C, RC	7	4.6	1-9
19	Placenta	1	1	IDT	F	8	6.9	2-9

*I = iododeoxyuridine; ID = I + 2-deoxy-d-glucose; IDT = ID + different temperature incubation.

†C = cocultivation; RC = reverse C; F = fusion; RF = reverse F; Inoc = frozen tissue inoculated.

‡PC = 6- to 10-week products of conception.

Kettering Cancer Center, New York, NY). Additional lines were used later: dog thymus (no. 8155), human lymphoblastoid (NCo-37; both obtained from Dr S. A. Mayyasi, Pfizer, Maywood, NJ); non-transformed rat kidney NRK line (parent of the H-NRK), feral mouse (III 6A; both obtained from Dr T. Pincus); and human xeroderma pigmentosum skin (CRL 1201; American Type Culture Collection). The lines were selected for their ability to replicate known type C viruses, and were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (DMEM-10) as described (Phillips *et al.*, 1976).

VIRUS ISOLATION

Enhancement of type C virus replication was attempted by adding 0.0005 mM dexamethasone (DXM) to the SLE cell culture medium for 1 week before testing. Virus induction was attempted by adding 5-iododeoxyuridine (IDU) at 30 µg/ml medium (Phillips *et al.*, 1976) and in later experiments 30 mM 2-deoxy-d-glucose (DG; Calbiochem, La Jolla, CA) to replicate SLE cultures (Prochownik *et al.*, 1976). One replicate with each inducer was incubated at 34°C, 37°C, and 40°C, to induce possible temperature-sensitive mutants (Stephenson *et al.*, 1972) for 24 hours before testing (Table 1).

The SLE and heterologous cell mixing methods evolved during the project (Table 1). All were done in 75 cm² plastic flasks (Falcon or Corning), except those with xeroderma pigmentosum cells were done

in 150 mm plastic dishes (Falcon) and the cells ultraviolet-irradiated (45 erg/s per mm²; 4.5 µJ/s) to induce DNA breaks (Robbins *et al.*, 1974) just before use. Initially, cocultivation (Phillips *et al.*, 1976) was done (8 experiments) by resuspending trypsinised SLE cells at approximately 10⁵ cells/ml (range 0.4-50 × 10⁵) in DMEM-10-DXM, placing 10 ml on 50-80% confluent monolayers of each heterologous cell line and incubating at 37°C. Reverse cocultivation was done (8 experiments) when the SLE cells were older and not growing rapidly; 10 ml of each heterologous cell line, trypsinised and resuspended as above (0.4-7 × 10⁵ cells/ml), was placed on 20-60% confluent SLE cell monolayers and incubated.

Subsequently, cell fusion (Vigier, 1973) was done (5 experiments) by placing 0.5 ml of trypsinised SLE cells (0.4-2.8 × 10⁶ cells resuspended in cold buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.25 mM cupric acetate) to enhance fusion (Wainberg *et al.*, 1973)) on 80-100% confluent monolayers of each heterologous cell line, adding 1 ml cold inactivated Sendai virus (diluted 1:10 in fusion buffer to approximately 10⁴ haemagglutination U/ml; Connaught Labs, Willowdale, Ontario) to each, mixing, incubating (37°C, 40 min), feeding DMEM-10-DXM, and incubating at 37°C. Reverse fusion was done (5 experiments) by placing 0.5 ml of each heterologous cell line (0.3-3.1 × 10⁶ cells resuspended as above) on 20-60% confluent SLE monolayers, adding Sendai virus, mixing, incubating, and feeding as above.

Frozen SLE tissues (5 experiments) were minced, homogenised in 5–10 volumes of cold Hanks's balanced salt solution with antibiotics, clarified (900 g, 20 min, 4°C), the supernatant filtered (0.45 µm; Millipore, Bedford, MA), 2 ml inoculated on each heterologous cell line previously treated with DEAE-dextran (25 µg/ml, 30 min, 37°C; Phillips *et al.*, 1976), incubated (1 h, 37°C), and fed DMEM-10-DXM.

Each SLE tissue was tested by one or two of the above methods on 4–10 different heterologous cell lines (mean 7.3, Table 1). When enough were available, SLE cells were also tested alone. Every third experiment (nine times in all), a control set of heterologous cell lines alone was handled in the same way as that experiment.

Subsequently, both SLE and control experiments were incubated at 37°C and examined twice a week for cell transformation or cytopathic effect. The medium was harvested, clarified (2000 g, 15 min, 4°C), and frozen at –70°C for later RDDP assay, and the cultures refed DMEM-10-DXM with antibiotics. The experiments were subcultured every 2 weeks for 1–7.5 months (mean 4.5 months): an average of 6.7 times in the SLE experiments (range 2.8–12, Table 1), and 5.3 times in the control sets. Variability in numbers of heterologous lines and of subcultures was due to quantity of SLE cells available, loss to contamination, and updating of methodology. During the entire project, 2251 subcultures were done. Virus induction was again attempted at intervals in most experiments by adding IDU and/or DG for 24 hours before subculture. Isolation of temperature-sensitive mutant virus (Stephenson *et al.*, 1972) was attempted by incubating the fourth subculture of all fusion experiments (Table 1) at 34°C, and the fifth at 40°C.

RDDP ASSAY

Supernatant medium harvested from rapidly growing cells was tested for RDDP (Stephenson *et al.*, 1972) routinely after every other subculture. The medium was concentrated 45-fold by ultracentrifugation (30 000 g, 90 min, 4°C), the pellets resuspended overnight at 4°C in 0.2 ml buffer (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl), and 50 µl of sample mixed with 50 µl of reaction mixture to contain the following final concentrations: 50 mM Tris-HCl, pH 7.8, 60 mM KCl, 0.2 mM manganese acetate, 2 mM dithiothreitol (Calbiochem), 0.1% triton X-100 (Calbiochem), 0.02 ²⁶⁰A units poly(rA)·d(pT)₁₂ (previously reannealed at 50°C in 50 mM Tris-HCl pH 7.3; PL Biochemicals, Milwaukee, WI), 0.02 mM 2'-deoxy-thymidine 5'-triphosphate (PL Biochemicals), and 5 µCi ³H-methyl thymidine 5'-triphosphate (³H-TTP; 40–60 Ci/mM; New England

Nuclear, Boston, MA). After incubation (37°C, 60 min), trichloroacetic acid precipitates were collected on 0.45 µm filters, washed, and ³H cpm measured (Phillips *et al.*, 1976). All samples were tested in triplicate. The positive control was unconcentrated medium from Moloney murine leukaemia virus-infected culture (kindly provided by T. Pincus). RDDP activity was calculated as pmol ³H-TTP incorporated into acid-precipitable material per ml of original sample.

Sixty-two assays were done and typically the positive virus control was 1000–2000 pmol ³H-TTP/ml. Clearly negative samples were <0.2 pmol ³H-TTP/ml; 0.2–0.9 pmol/ml was equivocal and other harvests from the same culture tested; 1 pmol/ml or greater was positive when confirmed on other harvests.

VIRUS CHARACTERISATION

Transmissibility was determined by inoculating 1 ml frozen medium from RDDP-positive or negative cultures onto DEAE-dextran pretreated flasks of rabbit, mink, bat, horse, monkey, H-NRK, dog, and human skin cells, incubating (1 h, 37°C), feeding DMEM-10-DXM, and subculturing a mean of 7.3 times (range 1–10) at 1- to 2-week intervals with IDU and DG added before the first and second. The cultures were examined microscopically and every other subculture tested for RDDP. A second experiment was done similarly except that horse and human skin cells were replaced by mouse, NRK, and Kirsten murine sarcoma virus-transformed non-virus-producing mink lung cells (K-mink; from Dr A. Albino), 60 mm multidishes (Linbro) were used, 4 ml fresh medium from RDDP-positive or negative cultures was inoculated per dish, and incubated overnight before feeding; no DG was used. A mean of 17.3 subcultures (range 8–19) was done at 1 week intervals. Every third subculture was tested for RDDP and, after subcultures, 2, 5, and 16 the inoculated and control H-NRK and K-mink lines were tested for rescue of murine sarcoma virus (MSV) by focus formation (Levy *et al.*, 1975). Fresh clarified medium from these cultures was inoculated as above onto pretreated NRK or mink lung dishes respectively, incubated, periodically observed microscopically for foci of MSV-transformed cells, 3–6 subcultures done, and the last tested for RDDP. Uninoculated controls were included.

Virus density was determined by ³H-uridine-labelling (10 µCi/ml) cultures for 18 hours, ultracentrifuging the medium on 15–60% sucrose gradients (SW27; 112 000 g, 3 h, 4°C), and collecting 1 ml fractions for ³H cpm and density measurements. Co-sedimentation of RDDP was determined by assaying fractions from similar unlabelled cultures;

a positive murine leukaemia virus control (Dr A. Albino) was included (Phillips *et al.*, 1976).

Electronmicroscopy (Dr H. N. Sarkar) was performed on pelleted culture medium and thin sections of cultured cells. Antigenic characterisation was performed (Dr H. P. Charman) on medium samples concentrated as for the RDDP assay and resuspended in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl buffer with 0.2% bovine serum albumin, using complement-fixation with antisera against the interspecies and species-specific determinants of rat and other type C viruses (Oroszlan *et al.*, 1972; Charman *et al.*, 1976). Mycoplasma tests were performed (Microbiological Associates, Bethesda, MD) on cells cultured without antibiotics for 3 subcultures, which were scraped into their medium, coded, and frozen at -70°C for later testing.

Results

Cell transformation or cytopathic effect was not observed in either SLE or control experiments. 13 of 737 harvests from SLE experiments were positive for RDDP (Table 2). Control harvests were only tested when the SLE were equivocal or positive; 9 of 75 were positive (Table 2). Of the positive samples, 4 SLE and 5 controls were from a cell line obtained from another laboratory already infected with murine leukaemia virus. The other 9 positive SLE samples were all low positive (1–10 pmol/ml; Table 2) and from the transformed non-MSV-producing rat cell lines (H-NRK) in 3 experiments (no. 7, 8B, 16). The 10 other lines in these experiments were negative. Experiments no. 7 and 16 were started together using the same H-NRK culture; both became positive after 5 subcultures but were lost after the sixth due to an incubator overheating. The H-NRK line in experiment no. 8B became equivocal (0.3–0.8 pmol/ml) after 4 subcultures and positive (1.8–3.6 pmol/ml) after 8. It was further subcultured and this 8B virus isolate characterised as described below. The H-NRK line in a simultaneous experiment (no. 8A, with kidney from the same patient) also became equivocal after 5 subcultures, but was lost to contamination after the seventh. The H-NRK lines in 9 other experiments and 2 control sets during this 3-month period gave sporadic equivocal results; three and one of these, respectively, were available for prolonged subculture as described below. The other 4 positive control samples (Table 2) all came from the original parent NRK line in a later transmission experiment. It and the experimental NRK line, then negative, were also further subcultured. Thus, except for the virus-contaminated mouse line, all the RDDP-positive harvests came from rat lines in both SLE experiments and, later, control sets.

Table 2 RDDP levels in culture harvests from SLE type C virus isolation project

Type of experiment (no.)	No. of samples tested: RDDP level (pmol $^3\text{H-TTP}$ incorporated/ml)			
	Negative	1–10	>10–100	>100
SLE experiments (31)	724	11	2	—
Control sets (9)	66	1	6	2
Prolonged subculturing				
Experiments (14)	54	33	32	16
Controls (6)	27	6	6	
Virus characterisation (5)	163	16	1	6

$^3\text{H-TTP}$ = ^3H -methyl thymidine 5'-triphosphate.

With prolonged subculturing the RDDP level of the 8B H-NRK line increased progressively. Up to the twelfth subculture they were generally < 1 pmol/ml, thereafter increasing to as high as 352.5 pmol/ml. All the H-NRK lines were derived from a single culture provided by another laboratory. This was subcultured 8 times before experiment no. 8B and thus a total of 16 times when it became RDDP positive there. The H-NRK lines in experiments, no. 7 and 16 became positive after 18 total subcultures, but 37 other experimental and control H-NRK lines were negative (Table 3) for 3–39 total subcultures (mean 18.4) during the regular experiments. Besides two 8B sublines, 13 other H-NRK lines were subcultured for prolonged periods to determine the frequency of virus production. Three experimental and 2 controls became positive (Table 3) after 27–38 total subcultures. The rest remained negative up to termination after 38–48 total subcultures. Although DG is now known to be an inducer of endogenous rat type C virus (Prochownik *et al.*, 1976), no direct relationship to this or other manipulations was apparent. Virus induction appeared to be a random event increasing in frequency with prolonged subculturing.

The multiple manipulations in these experiments raised the possibility the H-NRK lines might have been interchanged with other cell lines. They contained a marker, the defective MSV genome, that could be demonstrated by rescue with helper murine leukaemia virus, and which was not present in the other lines except for the morphologically distinguishable K-mink. Therefore, before termination the H-NRK lines, RDDP-positive and negative were infected with murine leukaemia virus grown in mink cells, subcultured once, and harvested medium tested for focus formation on mink cells. All were positive, thus proving they were H-NRK cells.

Some NRK lines (original parent of the H-NRK) also became positive: one control during regular subculturing as mentioned previously, and 2 experimental lines during prolonged subculturing (Table 3).

Table 3 Type C virus release by MSV-transformed and nontransformed rat and mink cells: comparison of experimental and control lines during regular subculturing and with further prolonged subculturing

Cell line	No. RDDP + lines/total lines			
	Regular subculturing		Prolonged subculturing	
	Experimental	Control	Experimental	Control
H-NRK	3/28	0/12	5/11	2/4
NRK	0/5	1/4	2/2	1/1
Mink & K-mink	0/32	0/15	0/1	0/1

In contrast, none of 47 experimental and control mink and K-mink (also containing the MSV genome) did so (Table 3). Thus presence of the defective MSV genome in the cell lines did not influence their becoming RDDP-positive.

Although the accumulated data suggested the 8B virus isolate was of rat origin, further experiments were done to define its precise nature and to exclude as far as possible the presence of any human type C virus determinants. Reisolation from the original spleen tissue was attempted in a repeat experiment done like the original one (Table 1), but with 16 subcultures for all lines and 28 for the two H-NRK flasks inoculated. This and the simultaneous control set were negative throughout. Like other rat type C viruses (Prochownik *et al.*, 1976), the 8B isolate was not transmissible to other cells, including rat, in two experiments using the RDDP assay for detection, nor was the virus transforming for any cell line, including NRK and mink. Under the conditions used, the isolate did not rescue the MSV genome from either H-NRK or K-mink cells: harvests from these cells previously inoculated with the isolate did not induce transformed cell foci on NRK or mink cells. Typical for type C viruses, the ^3H -uridine-labelled 8B isolate banded at 1.14–1.16 g/ml in sucrose gradients, as did its RDDP activity (Fig. 1). Morphologically, it was a typical type C virus (Fig. 2). Mycoplasma tests on cells and medium of the 8B isolate were negative. Antigenically, pelleted 8B virus and the isolates from two other RDDP-positive H-NRK lines (one experimental, one control) reacted in complement-fixation tests only with antisera against type C interspecies antigen and against rat type C species-specific antigen. Their titres with these antisera ranged from 1:4 to 1:64, and they were negative (<1:2) with antisera against feline (RD-114), baboon, and mouse type C species-specific antigens. Pellets from RDDP-negative H-NRK cultures were negative with all the antisera. The 8B isolate was thus firmly identified as a type C virus having the biological and antigenic characteristics of endogenous rat type C virus.

Fig. 1 Sedimentation in 15–60% sucrose gradients of type C virus isolated from experiment no. 8B. (A) ^3H -uridine-labelled virus; (B) RNA-directed DNA polymerase activity of unlabelled virus. Fractions obtained by bottom puncture after ultracentrifugation at 112 000 g for 3 hours.

Discussion

The hypothesis that a virus is involved in SLE is largely based on immunologically-mediated diseases caused by viruses in animals. The closely analogous model, NZ mouse disease, where an endogenous type C virus is involved, has stimulated recent studies of type C viruses in SLE. Lewis *et al.* (1974) first used indirect immunofluorescence to show the possibility of a type C-related antigen on rare peripheral blood lymphocytes from SLE patients, but this antigen has not been further defined. Strand and August (1974) then used competition radioimmunoassay to try to show type C-related antigens in SLE tissue extracts, although lower levels were also found in normal tissues. The contrast with a number of other negative studies of normal, neoplastic, and SLE tissues is not readily explained (August and Strand, 1977). Subsequently, Mellors and Mellors (1976) found a possibly type C-related antigen in renal glomeruli of one SLE patient using indirect immunofluorescence and specific antisera against type C viral proteins. Absorption studies showed the specificity of the reaction, that the antigen seemed most closely related to the endogenous feline virus, and the presence of a similar antigen in an extract of the patient's spleen. The initial report was convincing, but the antiviral antiserum apparently reacted with only about 10% of glomeruli, and those in a focal fashion, and the reaction was weaker in two additional positive SLE patients (in Shulman *et al.*, 1978).

Fig. 2 Electronmicrographs of type C virus isolated from H-NRK rat cultures. (a) Thin section of cells from experiment no. 8B showing budding (bc), immature (ic), and mature (mc) virus particles. Uranyl acetate and lead citrate. $\times 50\ 000$. (b) Typical type C particles in pelleted medium from experiment no. 15, with small fragment of cell debris (d). Negative stain, phosphotungstic acid, pH 7.4. $\times 54\ 000$ (kindly provided by Dr H. Sarkar).

Panem *et al.* (1976) found antigens possibly related to a putative human type C virus isolate in glomeruli from all of 11 SLE patients by immunofluorescence. Although control kidneys were negative, the antiviral specificity of the antiserum was not rigorously demonstrated; in particular, absorption was not done with uninfected cells of the same or similar kind used to produce the viral immunogen. The specificity of such antisera is a problem because the immunogen is derived from virus grown in mammalian cell cultures and, even after purification, may contain both cellular and medium components. The antisera then have other nonvirus reactivities, making rigorous absorption and proper controls necessary to assure specificity. Lastly, a more complex proposal was made of the co-operation of measles and type C viruses in producing SLE, supported by data suggesting SLE leucocytes contain both type C virus RDDP and DNA complementary to measles RNA (Aleksberova *et al.*, 1975), a finding not confirmed to date (in Shulman *et al.*, 1978).

These five studies suggest that type C viruses are involved in some cases of SLE, but the negative evidence is considerable. Type C virus-like particles

were found by electronmicroscopy with similar frequency in both normal and SLE placenta (Imamura, *et al.* 1976). Type C-related antigens were not found by radioimmunoassay in crude extracts of 14 tissues, some containing virus-like particles from 10 SLE patients. In spite of the use of protease inhibitors, one tissue still gave a false-positive result due to protease digestion of the radiolabelled virus antigen (Charman and Phillips, 1977). Markenson *et al.* (1976) used lactoperoxidase labelling and various type C antisera to detect a probable virus-related antigen on peripheral blood T cells, but this was found in both SLE and normals. There is disagreement about the existence in human sera of natural antibodies to lower mammalian type C viruses; when antiviral reactivity has been found, its specificity for virus components alone and its antibody nature are still uncertain. In any case, several studies included SLE patients who differed little from other human populations (reviewed in Phillips and Christian, 1978).

The actual origin of the type C viruses isolated from human material is also uncertain. These closely resemble the endogenous baboon and simian sarcoma viruses. However, it is clear the posulated human

endogenous virus has not been isolated (Charman *et al.*, 1976; Okabe *et al.*, 1976; Panem *et al.*, 1977; Stephenson and Aaronson, 1976; 1977; Todaro, 1975; Todaro *et al.*, 1976; Wong-Staal *et al.*, 1976). By analogy with NZ mouse disease, this should be the kind of virus involved in SLE, where it might be less repressed by genetic controls and so more easily isolated. Our study is the second reported attempt to isolate type C viruses from SLE. The earlier negative study of 8 tissues used less sensitive methods, relying principally on ultracentrifugal separation of radiolabelled virus for detection (Phillips *et al.*, 1976), whereas this study used more sensitive methods for isolating and detecting mammalian type C viruses, particularly the endogenous baboon virus.

Since the factors affecting type C expression in man are unknown, we included patients with varying disease manifestations, duration, activity, and treatment, and various tissues likely to contain type C viruses: kidney, spleen, placenta. The baboon virus was first isolated from placenta (Todaro *et al.*, 1974), and two placentas in this study (no. 5, 15) did contain virus-like particles (Imamura *et al.*, 1976). Prolonged cell culture may induce virus replication, but can also select nonpermissive cells; therefore SLE tissue extracts and both short- and long-cultured SLE cells were tested. We used chemical inducers and hormone enhancers of virus replication and cocultivation, cell fusion, or DEAE-dextran to aid viral penetration into possibly permissive heterologous and human cell lines. The latter were known to be permissive for both the endogenous baboon and simian sarcoma viruses, as well as other type C virus replication.

The mixed SLE and heterologous cells were subcultured frequently, often with additional chemical induction, and for prolonged periods to promote virus replication (Stephenson *et al.*, 1972; Vigier 1973; Todaro *et al.*, 1974, 1975, 1976; Levy *et al.*, 1975; Phillips *et al.*, 1976; Prochownik *et al.*, 1976; Panem *et al.*, 1977). Ultraviolet-irradiated human xeroderma pigmentosum cells were used since viral DNA might be more readily incorporated into their slowly-repaired DNA (Robbins *et al.*, 1974). Incubation at temperatures other than 37°C was used as possibly permissive for temperature-sensitive virus mutants (Stephenson *et al.*, 1972). For type C virus detection, we relied primarily on the RDDP assay. Morphological change was also monitored, although newly isolated type C viruses are generally not cytopathic or transforming. RDDP assay of supernatant culture medium has been a sensitive and specific method for detecting efficiently-replicating type C viruses. Its lower limit of sensitivity is in the range of 10⁴ particles; with the concentration

procedure used, levels of 10³ particles or greater per ml of medium would have been detected. Such levels are readily reached with prolonged subculturing in a virus-permissive system (Stephenson *et al.*, 1972; Todaro *et al.*, 1974, 1975; Phillips *et al.*, 1976; Prochownik *et al.*, 1976).

The methods were successful in isolating and detecting type C viruses. A contaminating virus introduced elsewhere into a stock cell line was readily recognised, but more significantly, an endogenous type C virus integrated in a 'normal' cell genome was induced and detected. This was fully characterised as rat type C virus: sporadically present in both inoculated and control rat cell cultures, induced by chemical treatment and prolonged subculture, not infectious for rat or other species cells, and not transforming. The intact virus and its RDDP enzyme banded at 1.15 g/ml in sucrose and it had typical type C virus morphology (Lennette and Cremer, 1975; Prochownik *et al.*, 1976; Rasheed *et al.*, 1976). Antigenically it had type C interspecies, but only rat species-specific, antigens (Charman *et al.*, 1976; Oroszlan *et al.*, 1972). There was thus no evidence of anything other than rat virus characteristics (Sherr *et al.*, 1976).

Since the methods were appropriate for other mammalian type C viruses, why was a human virus not isolated? Possible explanations are that such a virus does not exist, or is not involved in SLE, or is defective and unable to replicate. Alternatively, complete virus might be present or able to be isolated only in rare patients, certain tissues, or at certain times. Unlike New Zealand mice, SLE patients are not genetically homogeneous. Virus expression might vary from patient to patient, as was generally found in the previous immunological studies, but the patients and tissues studied here were also varied. It is most likely that the experimental conditions were not permissive for human type C virus replication, at least not efficiently enough to be detected. The appropriate conditions for isolating the postulated endogenous human virus thus remain unknown (Todaro *et al.*, 1976).

The evidence does not permit a definitive statement about the role of type C viruses in SLE, but virus expression is apparently not as enhanced as in NZ mouse disease. This conclusion is qualified by the use of various virus detection methods, specificity, and particularly sensitivity. The postulated endogenous human type C virus may be only distantly related antigenically to other type C viruses. Then detection methods using lower mammalian virus reagents would be very insensitive for the human virus. Recently isolated examples of such distantly related reoviruses include the equine infectious anaemia virus, and the bovine leukaemia, and deer type C

viruses. These probably would not have been detected in their host species with the reagents used in the various SLE studies. Furthermore, as mentioned above, human populations are not genetically homogeneous. Thus, given that genetic factors are involved, SLE patients should have heterogeneous manifestations, probably including variable type C expression. The relatively insensitive current methods of detection might then only be positive in certain patients (Markenson and Phillips, 1978).

Although further attempts to isolate type C viruses from SLE seem warranted, this study illustrates several of the problems. First, even in a laboratory where no known type C viruses are cultured, contaminants can be introduced by cell cultures and materials obtained elsewhere. Second, since all mammalian cells are postulated to have integrated endogenous type C virus genomes (Todaro, 1975), the heterologous cell lines widely used for isolation attempts may also contain their own virus genomes. The latter may then be isolated when appropriate conditions are used, rather than the human virus being sought.

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