intensity I_L. If the polychromator (*8*) wavelength range $\Delta\lambda$ is set to span the absorption edge of a heavy element in the crystal (such as the Fe K edge in hemoglobin or the Au L_{III} edge of an Au–heavy atom derivative of a protein), the maximum variation in $|\mathbf{F}(\mathbf{k},\lambda)|^2$ is then obtained as an aid in phase determination (*2*) through the sharp variation of the anomalous scattering factors f' and f'' in the immediate vicinity of the absorption edge. Variation in the absorption component of I_L, namely $T(\lambda,\mathbf{r})$, is also at a maximum, but this is an unavoidable complication of all multiple wavelength techniques (*9*).

The x-ray Laue technique apparently has not been used for protein crystal analysis, although polychromatic x-rays have been advocated for low-angle scattering measurements of macromolecules in solution (*10*). A related neutron Laue technique (*11*) has been applied to a myoglobin crystal (*12*); however, the full spectrum of thermal neutrons was used with a $\Delta\lambda/\lambda_1$ value of approximately 3, so that many Laue reflections contained contributions from multiple structure factors. Although it was believed (*11*) that accurate individual structure factors could be isolated by deconvolution or Fourier chopping (*12*), the technique has not been widely used (*13*).

The x-ray Laue diffraction technique for macromolecule analysis has four advantages over monochromatic radiation techniques: (i) optimal use of the naturally polychromatic synchrotron radiation spectrum, (ii) reduction in exposure time, (iii) direct production of integrated diffraction intensities with a stationary crystal (*14*), and (iv) simultaneous recording of many thousands of reflections. These advantages apply to both static and kinetic experiments. The latter could provide information (*4*) on the structural changes that occur during biochemical reactions—that is, for time-resolved crystallography.

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8. The term "polychromator" describes the arrangement of mirrors and absorbers making up a wide bandpass filter of the synchrotron x-radiation.
9. An alternative approach to the collection of diffraction data at multiple wavelengths has been described [U. W. Arndt, T. J. Greenhough, J. R. Helliwell, J. A. K. Howard, S. A. Rule, A. W. Thompson, *Nature (London)* **298**, 835 (1982)]. The energy resolution of our technique is described in (*4*); that of U. W. Arndt *et al.* may prove superior.
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Association of Parvoviruses with Rheumatoid Arthritis of Humans

Abstract. *A small virus resembling parvoviruses in its morphological and physicochemical properties was derived from synovial tissue of a patient with severe rheumatoid arthritis. This virus, designated RA-1, elicits a syndrome in neonatal mice that includes neurological disturbances, permanent crippling of limbs, dwarfism, alopecia, blepharitis, "masking," and a rigid curvature of the thoracic spine. Polyclonal antibodies against RA-1 display high virus neutralizing activity and in immunoassays detect reactive antigen in synovial cells from different rheumatoid arthritis patients but not persons with osteoarthritis. Putative parvoviruses isolated from several other rheumatoid arthritis patients are only weakly pathogenic for newborn mice but can generate RA-1 virus–specific antigens in tissues of these animals. It has not been established that RA-1 and existing parvoviruses of mammalian species are related.*

The etiology of chronic rheumatoid arthritis (RA) of humans has eluded identification since the first description of this insidious disease by Sir Alfred Baring Garrod in the 19th century (*1*). Infectious agents thought to be associated with RA have included bacteria, mycoplasma, viruses, and viroids (*2–4*). Conventional viruses are known to produce arthritides of humans which are usually of relatively short duration, cause no tissue necrosis or permanent disability, and require only symptomatic treatment (*5, 6*). The one example of a viral pathogen causing chronic arthritis of a mammalian host is the caprine arthritis-encephalitis retrovirus that elicits a proliferative synovitis and periarthritis in older goats (*7*). It was recently reported (*8*) that parvovirus-like agents can be isolated from the synovial tissue of patients with severe RA disease including the unidentified agent originally described by Godzeski *et al.* (*9*). Here we report the salient findings of a 3-year collaborative study leading to the recognition of these agents as viruses with unusual properties and a yet undefined link with human RA.

Long-term cultivation of rheumatoid synovial cells with WI-38 human lung fibroblasts was accompanied by the transient appearance of microfoci of piled-up, aggregated cells; electron microscopic examination of these cells revealed rare intracytoplasmic budding particles with long surface projections. Subsequent inoculation of culture extracts into brains of suckling mice led to the emergence of an agent that was lethal in mice and, on the basis of inconclusive evidence, was tentatively regarded as an enveloped RNA virus (*9*). This agent, hereafter called RA-1 virus, is identified in the present report as a DNA parvovirus on the basis of results from completed virological studies and ongoing biochemical work. More than 10,000 newborn mice have been used to date for the production, bioassay, and characterization of mouse lethal RA-1 virus and other putative isolates because a permissive cell culture system has not been found (*10*).

Table 1. Response of newborn mice to serial brain passage of synovial cell extracts from different RA patients. All isolates, derived from synovectomies of the knee or other joints, were either directly extracted for mouse brain inoculation or cultivated in vitro for several passages before extraction. Symptoms included marked runting (dwarfism) obvious within 30 days after neonatal infection, transient bloating of the abdominal cavity, skin disorders (for example, alopecia), and scattered mortality with or without prior neurological disturbances. Permanent paralysis of the limbs was produced only by isolate RA-1. At a given passage level not all of the positive symptoms listed were expressed for isolates other than RA-1. Controls included serial brain passage of extracts of synovial cells from DJD patients. The total observation period was 60 days or less.

Isolate	Total brain passages	Composite symptoms observed for				ELISA reactivity with RA-1 antibody*
		Dwarfism	Sporadic deaths	Bloating	Skin and hair disorders	
RA-1	18	+++	+++	+++	+++	+++
RA-3	7	+	++	++	+†	++
RA-6	5	+	+	+	+‡	+++
RA-7	4	+	−	−	−	+++
DJD-1	4	−	+	−	−	−

*Polyclonal rabbit antibody produced against RA-1 virus was used in immunoassays with Hirt extracts of brains from mice intracerebrally inoculated with a given isolate. †Rare face "masking." ‡Alopecia: −, no response; +++, strong response.

Intracerebral inoculation of neonatal Swiss-Webster mice with cell-free brain extracts from RA-1–infected mice produces a central nervous system (CNS) disorder marked by early tremor, ataxia, convulsive movements, paralysis, and death in 5 to 10 days. Suitable extraction procedures have demonstrated that the virus infectivity titer of brain tissue from moribund animals reaches a median lethal dose (LD_{50}) of 10^8 or higher per gram. Infection of suckling mice by the intraperitoneal route results in less severe CNS disease with reduced mortality, and an extended latent period of about 10 to 18 days. Early signs of intraperitoneal disease include a transient bloating of the abdominal cavity in some animals, the appearance of wet matted fur, and occasional hyperactive motion. Progressive and permanent paralysis of one or more limbs first develops about 10 to 14 days after intraperitoneal infection (Fig. 1A). Surviving mice frequently show runting or dwarfism, a ruffled discolored coat, blepharitis, and sporadic loss of body hair (alopecia). Occasionally, they develop malformations of the teeth involving unarrested growth and "sabertooth" formation. Another rare consequence of virus infection is the development of a masklike pattern of hair on the face of alopecic mice; this pattern appears simultaneously in different litter mates about 28 days after neonatal infection and disappears in another 10 days. In some animals, including rats, a marked kyphosis of the thoracic and lumbar spine develops after intracerebral infection with RA-1 that mimics the response of neonatal mice to RA synovial cell extracts first described by Warren and co-workers (*11*) (Fig. 1B). Mice older than 15 days show resistance to infection, although in adult animals made susceptible by immunosuppressive drugs there is an abrupt onset of a fatal disease. Newborn hamsters and rats show a much lower frequency of infection with RA-1 and a latent period of 50 days or more.

Rigorous extraction techniques with Hirt detergent–high salt buffer (*12*) or chloroform-butanol solvent mixtures have shown that the infectivity of RA-1 virus from mouse brain tissue is highly stable. The RA-1 infectivity was not abolished by exposure to various proteases, nucleases, lipid solvents, low *p*H, prolonged ultrasonication, or heating at 56°C for several hours (*10*). That this pathogen was readily inactivated by moderate doses of ultraviolet radiation, common disinfectants, or highly alkaline buffers excluded it from currently recognized classes of exotic agents and "unconventional" viruses (*13*).

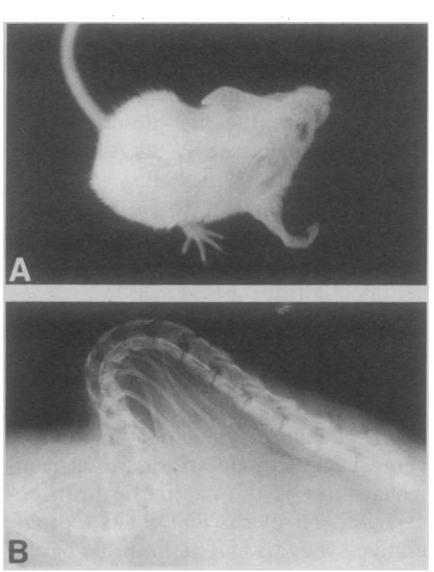


Fig. 1. Pathological effects of RA-1 virus in neonatal rodents. (A) Permanent crippling of the right front leg of a 38-day old Swiss mouse inoculated intraperitoneally at birth with RA-1 virus. The rigid paralysis and extension of the afflicted limb developed within 2 weeks after infection. (B) Roentgenogram showing marked curvature of the spine of a 56-day old Wistar-Lewis rat inoculated intracerebrally with virus 3 days after birth.

Sedimentation studies indicated that the infectivity of RA-1 was associated with particles slightly smaller than poliovirus virions. The unusual resistance of this isolate, its apparent small size, and its pathogenesis for neonatal rodents suggested that it might be a parvovirus. Accordingly, we detected hexagonal particles with an average diameter of about 24 nm and a buoyant density of 1.4 g/ml in isopycnic density gradients used for centrifugation of brain extracts from infected mice (Fig. 2). Gradient fractions containing these particles produced lethal disease in neonatal mice with the same symptoms caused by crude brain extracts from RA-1–infected animals. We were unable to detect 24-nm particles in brains of normal mice from our colony or mice intracerebrally inoculated with extracts of synovial cells from particles can be extracted for a single-stranded DNA which is approximately sive evidence for the identity of RA-1 as a parvovirus comes from our recent success in demonstrating that CsCl gradient fractions containing the infectious 24-nm particles can be extracted for a single–stranded DNA which is approximately 4.5 kilobases in size. Studies of the molecular properties of this DNA species with restriction enzymes indicate that its cleavage pattern is different from that of existing parvoviruses (*14*).

When rabbits were immunized with either crude Hirt extracts (supernatants) of RA-1–infected mouse brain or purified preparations of virus from the same source, they produced antisera with high virus-neutralizing activity. These antisera have allowed the use of sensitive enzyme-linked immunosorbent assays (ELISA) both for detecting antigenically related viruses in synovia of different RA patients and for understanding the possible relation of RA-1 virus to other viruses. Table 1 shows that synovial cell extracts from the representative human RA patients, but not from a DJD control, were positive for RA-1 antigen. Only RA-1 was virulent for mice at each serial passage; other synovial derivatives gave a sporadic and unpredictable response for one or more of the symptoms listed.

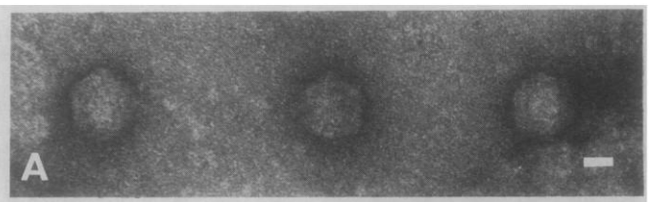
The relatively high virulence of RA-1 probably reflects a poorly understood adaptation process associated with its

long-term passage in tissue culture and in mice. Most of these animal experiments were of relatively short duration (30 to 60 days) and it is possible that the various clinical isolates tested require a long latent period for the expression of a more severe disease. It is important, however, that 13 of 14 separate synovial specimens from RA patients studied to date have generated antigen reactive in ELISA tests with RA-1 antibody. Table 2 shows that the amount of reactive antigen detected with highly dilute antiserum varied among these isolates. The eight osteoarthritis patients monitored for reactive antigen in their synovial cells after either cultivation in vitro or serial mouse brain passage of extracts have produced ELISA titers that are considered to be insignificant and nonspecific.

A critical question remaining was whether RA-1 virus and the other putative viral isolates of purported human synovium origin actually represent adventitious rodent parvoviruses that were activated and amplified as a consequence of serial mouse brain passage of our clinical specimens. Different lines of evidence argue against this possibility, including the findings that human RA synovial cells cultivated in vitro are positive in ELISA tests for RA-1–reactive antigen, in some cases at the primary passage (Table 3). With isolate RA-6, for example, synovial cells from the same human donor on separate occasions were positive for viral antigen following either serial mouse brain passage (Table 2) or cultivation in cell culture (Table 3).

Although more than 25 different cell lines tested do not support productive infections with RA-1 virus, we have found that some cells, such as Crandell feline kidney (CRFK), will produce RA-1–specific antigens detectable in ELISA tests when inoculated with RA-1 and other isolates. RA-1 virus antigen from CRFK cells does not react with antisera against several parvoviruses tested including type 2 human adeno-associated virus (AAV), canine AAV, Kilham rat virus (KRV), H1, minute virus of mice (MVM), Haden virus, LuIII virus, the Kirk agent, and B19 virus. Complement-fixation tests carried out with our RA-1 rabbit antiserum at Microbiological Associates (Bethesda, Maryland) gave negative results with antigens of rodent viruses including Theilers GPVII, pneumonia virus of mice, reovirus type 3, polyoma virus, Sendai virus, ectromelia virus, simian virus 5, pneumonitis K virus, MVM, and KRV. Finally, workers in England have independently shown that extracts of synovium from RA patients, but not osteoarthritis patients, contain small unidentified 12- to 14-nm particles that react with our RA-1 antibody in ELISA tests (*15*).

The relation of parvoviruses to human disease has remained obscure since the first recognition of these small DNA viruses in different animal species. It has been recently established that parvoviruses are associated with aplastic crises in chronic hemolytic anemias of humans and that these viruses appear to be widespread (*16*). Parvoviruses are insidious



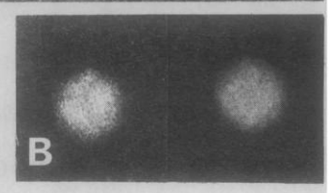


Fig. 2. Morphological appearance of RA-1 virus particles examined by electron microscopy. Hirt extracts of brain tissue from Swiss mice intracerebrally inoculated at birth with the virus were centrifuged through a layer of 30 percent sucrose-STES (0.1*M* NaCl, 0.05*M* tris-HCl, 0.001*M* EDTA, 1 percent Sarkosyl, *p*H 8) onto a cushion of CsCl (density 1.5 g/ml) in an SW-28 rotor at 25,000 rev/min for 5 hours. Material collected at the interface of the CsCl layer was centrifuged in a preformed CsCl gradient (density 1.4 g/ml) in an SW-55 rotor at 35,000 rev/min for 48 hours. Pelleted material from the gradient fraction corresponding to a density of 1.4 g/ml was negatively stained with 2 percent uranyl acetate (A) or 2 percent neutral phosphotungstic acid (B) and examined in a JEM-100 electron microscope at an instrumental magnification of 150,000 diameters and operating voltage of 100 kv. Magnification bar, 10 nm.

Table 2. ELISA reactivity of brain extracts from neonatal mice inoculated intracerebrally with different passage levels of putative viral isolates from synovial cells of RA patients. Brain extracts standardized for protein content were tested in immunoassays with serial dilutions of rabbit antiserum to RA-1 virus. These assays involved coating of microtiter plates with test antigen followed by sequential addition of RA-1 antibody, alkaline phosphatase–conjugated goat antiserum to rabbit globulin, and paranitrophenyl phosphate substrate. Serially passaged synovial extracts of patients with DJD were included as controls. Serum from nonimmunized rabbits gave negative results (data not shown).

RA-1 antiserum dilution	ELISA value with brain extract of mice inoculated with*		
	RA-1 (MB P16)	RA-3 (MB P5)	RA-6 (MB P4)†
1/10,000	0.930	0.648	0.884
1/20,000	0.336	0.175	0.530
1/40,000	0.583	0.067	0.421
1/80,000	0.374	0	0.173
1/160,000	0.299	0	0.082
1/320,000	0.145	0	0
1/640,000	0.063	0	0

*Values shown were derived by subtracting the "background" readings obtained in tests with brain extracts of mice used for passage of DJD synovium controls. Absorption was at 405 nm. †Fourth serial mouse brain passage of synovial membrane initially extracted with chloroform–isoamyl alcohol and obtained from the second of three consecutive synovectomies performed on patient RA-6.

Table 3. ELISA end-point titers obtained with extracts of cultured human synovial cells from two RA patients in reactions with antisera to RA-1 virus. Hirt extracts of cultures made after primary (P1) or single passage (P2) in vitro were run in standardized ELISA tests with rabbit antiserum to RA-1 (virus neutralization titer, >1:10,000). Extracts from cells of patients with degenerative joint disease (osteoarthritis) cultured under identical conditions served as controls.

RA-1 antiserum dilution	ELISA value obtained with extract*			
	RA-6 P1†	RA-6 P2†	RA-10 P1	RA-10 P2
1/40	0.037	0.306	0.227	0.260
1/80	0.006	0.192	0.336	0.014
1/160	0	0.207	0.099	0.071
1/320	0	0.108	0.121	0.041
1/640	0	0.086	0.091	0.032
1/1280	0	0.060	0.082	0.034
1/2560	0	0.028	0.052	0
1/5120	0	0	0.058	0

*All values were corrected by subtracting the readings obtained with the osteoarthritis control. Absorption was at 405 nm. †Cells obtained from third consecutive synovectomy of patient RA-6.

pathogens which are often "silent" in cell culture systems or laboratory animals and can also persist at the molecular level as integrated inserts in chromosomal DNA (*17, 18*). The significance of the parvoviruses described in this report in the genesis of human connective tissue disease remains to be resolved.

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Circadian Changes in Enzyme Concentration Account for Rhythm of Enzyme Activity in *Gonyaulax*

Abstract. *A circadian rhythm in the activity of luciferase is partly responsible for rhythmic bioluminescence in the dinoflagellate alga* Gonyaulax polyedra. *The cyclic activity of this enzyme can be attributed to a corresponding rhythm in the concentration of immunologically reactive luciferase protein. Hence protein turnover (synthesis or degradation or both) is used by the endogenous clock to control the daily rhythm of bioluminescence.*

In an effort to elucidate the biochemical basis of circadian rhythmicity (*1*), we studied the pathway by which the biological clock controls bioluminescence in the dinoflagellate alga *Gonyaulax polyedra*. In previous studies (*2, 3*) it was found that the in vitro activity of luciferase, the enzyme that controls the rate-limiting step in the bioluminescence reaction, is correlated with bioluminescence in vivo; both are rhythmic, even under constant environmental conditions. The 5- to 15-fold modulation in luciferase activity during the circadian cycle was shown to be due either to a covalent modification of the enzyme or to changes in the amount of enzyme (*3, 4*), and titration of enzyme activity with an inhibiting antibody to luciferase suggested that day luciferase and night luciferase were immunologically indistinguishable (*5*). These studies implied that the enzyme is synthesized or degraded (or both) cyclically.

However, proteolysis of luciferase (*4*) or inhibition of luciferase activity by a heat-stable component in crude *Gonyaulax* extracts (*6*) during the incubation of luciferase and antibody could confound interpretation of the titration results. Moreover, the possibility that luciferase compartmentation might undergo rhythmic changes was never ruled out [and luciferase is at least partially compartmentalized (*7*)]. To evaluate these possibilities and to quantify luciferase, we directly measured luciferase protein with antibody to luciferase. We found that luciferase is indeed modulated by the circadian clock; the magnitude of this change largely accounts for the rhythm of enzyme activity.

The polyclonal antibody we used inhibits luciferase activity in vitro (*5*). As shown in Figs. 1B and 2B, this antibody recognizes a single protein (molecular weight, 135,000) from crude extracts of *Gonyaulax*. The antibody also recognizes purified luciferase and the active proteolytic fragment of luciferase (molecular weight, 30,000 to 40,000), which is extractable under different conditions (*4*). We have little doubt, therefore, that the protein recognized by the antibody is luciferase.

Figure 1 depicts the daily cycle in activity and band density of luciferase from *Gonyaulax* cells maintained in an LD 12:12 cycle (12 hours of light followed by 12 hours of darkness). The amount of enzyme parallels the rhythm of enzyme activity from cells extracted every 3 hours during 51 hours in the cycle. To be sure that these rhythms are not merely driven by the LD cycle but are controlled by the endogenous clock, we performed the same experiment with cells maintained in constant dim light (LL). The results (Fig. 2) demonstrate that the circadian clock is indeed responsible for regulating turnover of this enzyme. Soluble protein extracts were used in the experiments represented in Figs. 1 and 2, but similar results were obtained with total protein extracts (which include organelles). Therefore, shuttling of luciferase between intracellular compartments is not responsible for the daily regulation of luciferase. The clock is controlling daily alterations in the total quantity of enzyme in the cell.

How large are these changes in the level of luciferase? The densitometry data in Figs. 1 and 2 suggest that the changes in enzyme level account for the change in activity, but this assay is valid only if the density is a linear function of protein concentration. Densitometry data corrected for linearity of the relation of density to protein (*8*) are compared in Table 1 for immunoblotted samples taken from the peak and trough of the oscillation. We also developed an enzyme-linked immunosorbent assay (ELISA) that allowed luciferase to be measured in micrograms per milligram of soluble protein (*9*). Table 1 shows that these methods of luciferase determination yield 6- to 16-fold night-to-day ratios of luciferase concentration corresponding with 9- to 34-fold activity ratios. The discrepancy between activity and concentration ratios might be due to inaccu-