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Biochemical Characterization of a Human Parvovirus

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SUMMARY

The buoyant density, nucleic acid, and proteins of the human serum parvovirus-like agent were investigated. Evidence is presented which suggests that the virus has genomic single-stranded DNA, and that complementary strands may be encapsidated in separate virions. Three proteins of 48000, 68000 and 80000 mol. wt. were found to co-purify with viral antigen at a density of 1·43 g/ml on CsCl gradients. On the basis of these properties it is suggested that this virus is a parvovirus.

A previously undescribed virus was found in human sera by Cossart and associates in 1975. On examination in the electron microscope, virus particles were seen, which on the basis of size and morphology were classified as parvovirus-like (Cossart *et al.*, 1975; Paver & Clarke, 1976; Anderson, 1982). Subsequent studies have associated this virus with febrile illness (Shneerson *et al.*, 1980), the aplastic crisis of sickle cell disease and hereditary spherocytosis (Serjeant *et al.*, 1981; Kelleher *et al.*, 1983), and most recently with erythema infectiosum (fifth disease) of childhood (Anderson *et al.*, 1983).

The molecular composition of this virus has not been investigated because it has not been possible to grow the virus in tissue culture, and because supplies of serum containing viral antigen are limited. For these reasons it was decided to investigate the proteins and nucleic acid of the virus by *in vitro* radiolabelling techniques which do not depend on tissue culture-grown virus. The serum and plasma samples containing parvovirus antigen used in this study were those of the Virus Reference Laboratory collection: B19 (Cossart *et al.*, 1975), JB (Kelleher *et al.*, 1983), Wi, Pr, Br I, Br II (Mortimer *et al.*, 1983) and were supplied by Dr B. J. Cohen. All six samples were analysed by CsCl banding, but only Wi and Br II were used for further DNA and protein analyses.

Initial experiments were performed to determine accurately the buoyant density of the serum virus in CsCl. These were possible because of the availability of a sensitive radioimmunoassay (RIA) for viral antigen (Cohen *et al.*, 1983). The six samples of sera were clarified by centrifugation through a Hemmings filter, prior to banding in CsCl. After fractionation, the position of the virus was determined, and the refractive index of each fraction measured to determine the density. The mean buoyant density of the virus was found to be 1·43 g/ml, with a range of 1·41 to 1·45. One such experiment, in which a sample of the serum containing the original virus isolate (B19) was analysed, is shown in Fig. 1. In addition to the main peak at 1·43 g/ml there was a reproducible shoulder at 1·39 g/ml, perhaps because of the presence of empty capsids. Thus, these gradients are a means of purifying the virus from serum. We have also found that combined potassium tartrate/glycerol gradients (Obijeski *et al.*, 1974; Ashley & Caul, 1982) provide a satisfactory separation.

Nucleic acid was extracted from the virus (from Br II plasma) by proteinase K treatment (100 μg/ml in 0·5% SDS for 15 min at 37 °C) prior to extraction with a 50:50:1 mix of phenol:chloroform:isoamyl alcohol, and recovered by ethanol precipitation. Because of the limited amount of specimen available, nucleic acids were detected by Southern blotting and hybridization, using a nick-translated probe prepared from viral nucleic acid. The nature of the nucleic acid was investigated by incubating it with various enzymes prior to electrophoresis on

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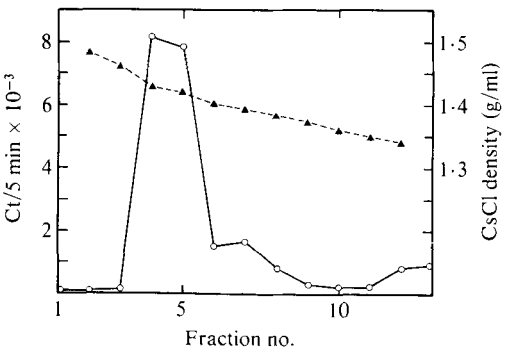

Fig. 1

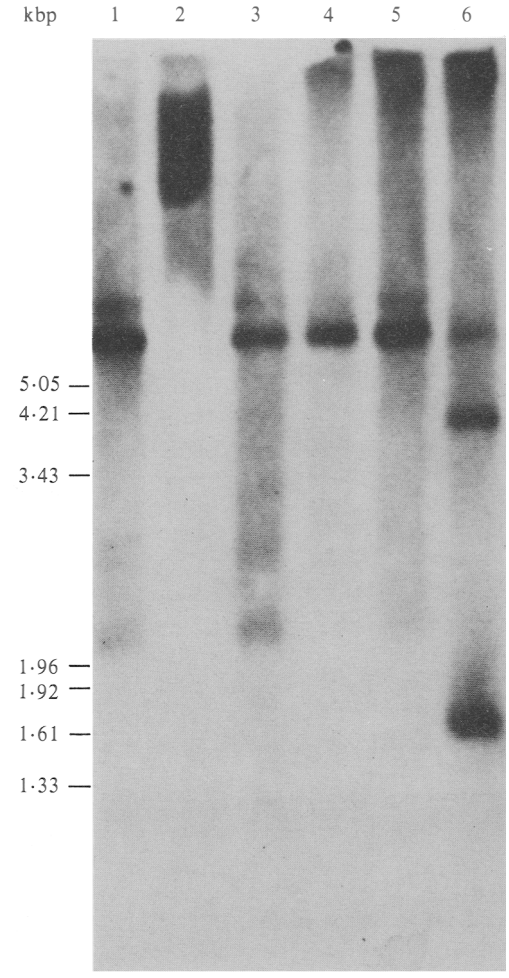

Fig. 2

Fig. 1. Centrifugation of virus in CsCl. One-hundred µl of B19 serum in 1·39 g/ml CsCl was centrifuged at 35000 rev/min for 24 h in a Beckman SW50.1 rotor. Fractions were collected under gravity. Density (▲) was determined by measuring the refractive index of fractions. Viral antigen (○) was determined using the antibody capture radioimmunoassay exactly as described by Cohen *et al.* (1983). Essentially, polystyrene beads coated with goat anti-human µ-chain and human anti-parvovirus IgM were sequentially reacted with test specimen, anti-human parvovirus monoclonal antibody, and ^{125}I-labelled sheep anti-mouse immunoglobulin. Binding of the label to the beads was monitored using a gamma counter.

Fig. 2. Effect of enzyme treatments on viral DNA. Lane 1, control; lane 2, incubation with 1 µg DNase I (bovine pancreas, Calbiochem-Behring) in 0·1 M-NaCl, 1 mM-$MgCl_2$, 20 mM-Tris–HCl pH 7·4; lane 3, incubation with 10 units RNase T1 and 1 µg RNase A in 20 mM-Tris–HCl, 2 mM-EDTA, pH 7·4; lane 4, incubation with *Eco*RI; lane 5, incubation with *Hin*dIII; lane 6, incubation with *Pst*I. All reactions were at 37 °C for 30 min, and restriction digests were performed under the conditions recommended by the supplier (Bethesda Research Laboratories). DNA was electrophoresed on horizontal gels of 1% (w/v) agarose in 40 mM-Tris–HCl, 20 mM-sodium acetate, 2 mM-EDTA, pH 7·0. The position of the *Eco*RI/*Hin*dIII digest of phage lambda DNA is indicated in kbp, and was determined visually under u.v. light after staining with ethidium bromide. Gels were blotted onto nitrocellulose following the procedure of Southern (1975). Hybridization of the filter, with ^{32}P-labelled DNA (Rigby *et al.*, 1977), was at 42 °C in 50% formamide, 3× saline sodium citrate (SSC), 50 mM-HEPES, 0·5 mg/ml yeast RNA, 10 µg/ml sheared salmon sperm DNA, supplemented with Denhardt's reagents (Denhardt, 1966). After hybridization, the filter was washed with decreasing concentrations of SSC to a final wash of 0·1× SSC. Autoradiography was performed at −70 °C using a DuPont intensifying screen.

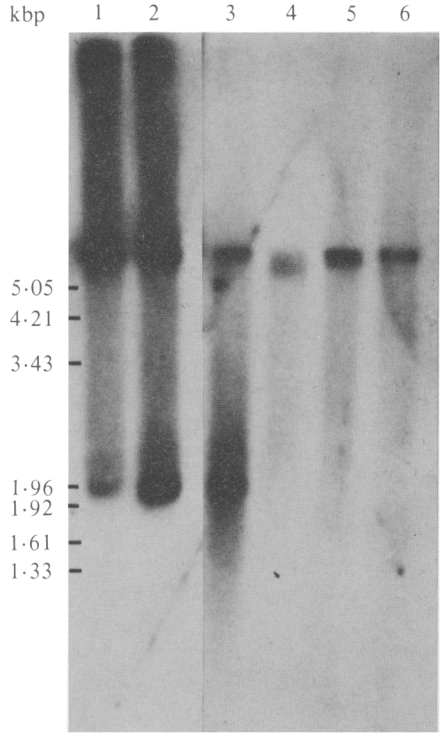

Fig. 3

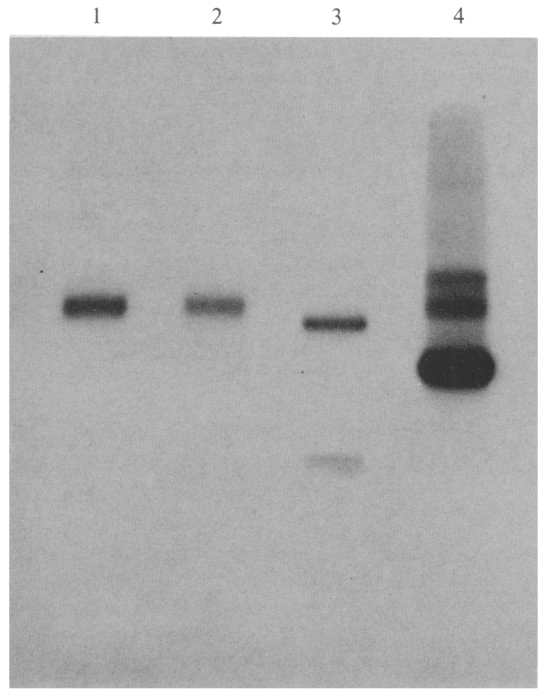

Fig. 4

Fig. 3. Effect of brief heat treatment and nuclease S1 on viral DNA. Lane 1, control; lane 2, DNA incubated at 70 °C for 5 min; lane 3, 0·1 unit S1; lane 4, 1 unit S1; lane 5, 10 units S1; lane 6, 100 units S1. Incubation was at 37 °C for 30 min in 0·2 M-NaCl, 0·05 M-sodium acetate, 1 mM-$ZnSO_4$, 0·5% glycerol, pH 4·5. The lambda markers were also heated at 70 °C for 5 min before electrophoresis.

Fig. 4. Polyacrylamide gel electrophoresis of ^{125}I-labelled proteins. Lane 1, serum containing no antigen; lane 2, serum containing antigen; lane 3, immune-precipitated antigen-positive serum; lane 4, gradient-purified virus. Proteins were iodinated using the 'Iodogen' reagent (Pierce Chemical Co., Rockford, Ill., U.S.A.) as described by Salacinski *et al.* (1979). Immune precipitation was performed by incubating viral antigen with 10 µl of human serum containing antibody to human parvovirus at 4 °C overnight, and then by precipitation with staphylococcal Protein A as described by Kessler (1975). After precipitation and recovery, antigen–antibody complexes were dissociated by heating at 95 °C for 3 min in a buffer containing 10% (w/v) SDS, 25% (v/v) 2-mercaptoethanol, 0·25 M-Tris–HCl pH 6·8, 20% glycerol and 0·002% bromophenol blue. Proteins were electrophoresed on 10% discontinuous polyacrylamide gels using a Tris–glycine buffer system (Laemmli, 1970). Molecular weight markers were visualized by staining with Coomassie Brilliant Blue, or, on occasion, radiolabelled markers were used. Gels were dried under vacuum and exposed to X-ray film.

1% agarose gels. Such an experiment is shown in Fig. 2. Control untreated nucleic acid (lane 1) gave a major band with an apparent size of 5·6 kilobase pairs (kbp), by comparison with an *Eco*RI/*Hin*dIII digest of phage lambda DNA. There was also a minor band of lower electrophoretic mobility (about 6·3 kbp), the nature of which is unclear. The 5·6 kbp band was demonstrated to be double-stranded DNA by its susceptibility to digestion with DNase (lane 2) but not with RNase (lane 3) under conditions in which both single- and double-stranded RNAs were cleaved. It was not cleaved by *Eco*RI or *Hin*dIII (lanes 4 and 5), but was cleaved once by *Pst*I to give 1·65 and 4 kbp fragments (lane 6), consistent with it being a linear rather than a circular form. The 5·6 kbp band is about twice the expected size of parvovirus DNA and could be explained if complementary single-strands were annealing during phenol extraction, a phenomenon often observed for parvovirus DNA. To test this explanation, the DNA was

analysed on gels before and after incubation at 70 °C for 5 min, treatment which might be expected to separate incomplete duplexes, and which did not separate lambda *Eco*RI/*Hin*dIII DNA fragments in control experiments. Fig. 3 (lane 2) shows that, following heat treatment, a species migrating with an apparent size of about 2 kbp was seen, and was evidently derived from the larger form. To determine the nature of this molecule, the DNA was incubated with the single-strand-specific nuclease S1. At increasing concentrations, the enzyme digested this band but not the 5·6 kbp band (Fig. 3, lanes 3 to 6), suggesting that the lower band was the single-stranded precursor of the upper duplex band. The migration of the lower band was compared to that of the parvovirus densonucleosis virus (DNV) single-stranded DNA (data not shown), and was found to be slightly higher, providing an independent estimate of its molecular weight, found to be $1{\cdot}7 \times 10^6$.

This evidence, while not as convincing as that obtainable if it were possible to culture the virus, is consistent with the hypothesis that positive and negative complementary single-stranded DNA, of approx. $1{\cdot}7 \times 10^6$ mol. wt., is packaged in separate virus particles. On extraction of DNA the strands are able to anneal to form double-stranded molecules of approximately twice the molecular weight ($3{\cdot}4 \times 10^6$) of the single-stranded form. Molecules of even lower electrophoretic mobility, as seen in Fig. 2, could be formed by hydrogen bonding of complementary terminal sequences, a characteristic of parvovirus DNA (Berns & Hauswirth, 1978). Such behaviour is typical of the DNA of the dependoviruses (adeno-associated viruses) and the densoviruses, although it is by no means confined to them, since the autonomous parvovirus LuIII also shows this property (Kelly *et al.*, 1977; Mayor & Kurstak, 1974; Muller & Siegl, 1983; Rose *et al.*, 1969).

The protein composition of the virus was investigated by radiolabelling gradient-purified preparations with ^{125}I. Fig. 4 shows the protein profile of such virus (lane 4). There is a heavily labelled band at 48000, and two others at 68000 and 80000 daltons respectively. The first two lanes show that if whole serum, either antigen-positive or -negative, was labelled, then, as expected, only albumin was visible. The third lane shows that immune precipitation of antigen-positive labelled sera with Protein A and antibody only allow the detection of the heavy chain of IgG. To determine whether the three proteins co-purifying with viral antigen were authentic virion components, the iodinated purified virus was immune-precipitated and the products analysed by electrophoresis (data not shown). Although iodinated proteins have poor antibody-binding properties, both the 48000 and the 68000 dalton proteins precipitated, suggesting that they are viral antigens. The 68000 protein co-migrated with serum albumin on polyacrylamide gels, and peptide mapping experiments were inconclusive. Proteins of these molecular weights have been described for other parvoviruses (Tattersall, 1978). The autonomous parvoviruses have been reported to have a major virion protein of 61000 to 69000 mol. wt., the dependoviruses one of 56000 to 69000, and the densoviruses one of 41000 to 49000 (Moore & Kelly, 1980; Tattersall, 1978; Tijssen & Kurstak, 1981).

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REFERENCES

ANDERSON, M. J. (1982). The emerging story of a human parvovirus-like agent. *Journal of Hygiene* **89**, 1–8.

ANDERSON, M. J., JONES, S. E., FISHER-HOCH, S. P., LEWIS, E., HALL, S. M., BARTLETT, C. L. R., COHEN, B. J., MORTIMER, P. P. & PEREIRA, M. S. (1983). The human parvovirus, the cause of erythema infectiosum (fifth disease)? *Lancet* i, 1378.

ASHLEY, C. R. & CAUL, E. O. (1982). Potassium tartrate–glycerol as a density gradient substrate for separation of small, round viruses from human feces. *Journal of Clinical Microbiology* **16**, 377–381.

BERNS, K. I. & HAUSWIRTH, W. W. (1978). Parvovirus DNA structure and replication. In *Replication of Mammalian Parvoviruses*, pp. 13–32. Edited by D. C. Ward & P. Tattersall. New York: Cold Spring Harbor Laboratory.

COHEN, B. J., MORTIMER, P. P. & PEREIRA, M. S. (1983). Diagnostic assays with monoclonal antibodies for the human serum 'parvovirus', B19. *Journal of Hygiene* **91**, 113–130.

COSSART, Y. E., FIELD, A. M., CANT, B. & WIDDOWS, D. (1975). Parvovirus-like particles in human sera. *Lancet* i, 72–73.

DENHARDT, D. T. (1966). A membrane filter technique for the detection of complementary DNA. *Biochemical and Biophysical Research Communications* **23**, 641–646.

KELLEHER, J. F., LUBAN, N. L. C., MORTIMER, P. P. & KANIMURA, T. (1983). The human serum 'parvovirus': a specific cause of aplastic crisis in hereditary spherocytosis. *Journal of Pediatrics* **102**, 720–722.

KELLY, D. C., BARWISE, A. H. & WALKER, I. O. (1977). DNA contained by two densonucleosis viruses. *Journal of Virology* **21**, 396–407.

KESSLER, S. W. (1975). Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody–antigen complexes with protein A. *Journal of Immunology* **115**, 1617–1624.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

MAYOR, H. D. & KURSTAK, E. (1974). Viruses with separately encapsidated complementary strands. In *Viruses, Evolution and Cancer*, pp. 55–78. Edited by E. Kurstak & K. Maramorosch. New York: Academic Press.

MOORE, N. F. & KELLY, D. C. (1980). Interrelationships of the proteins of two insect parvoviruses (densonucleosis virus types 1 and 2). *Intervirology* **14**, 160–166.

MORTIMER, P. P., HUMPHRIES, R. K., MOORE, J. G., PURCELL, R. H. & YOUNG, N. S. (1983). A human parvovirus-like virus inhibits haematopoietic colony formation *in vitro*. *Nature, London* **302**, 426–429.

MULLER, D. E. & SIEGL, G. (1983). Maturation of parvovirus LuIII in a subcellular system. I. Optimal conditions for *in vitro* synthesis and encapsidation of viral DNA. *Journal of General Virology* **64**, 1043–1054.

OBIJESKI, J. F., MARCHENKO, A. T., BISHOP, D. H. L., CANN, B. W. & MURPHY, F. A. (1974). Comparative electrophoretic analysis of the virus proteins of four rhabdoviruses. *Journal of General Virology* **22**, 21–33.

PAVER, W. K. & CLARKE, S. K. R. (1976). Comparison of human fecal and serum parvo-like viruses. *Journal of Clinical Microbiology* **4**, 67–70.

RIGBY, P. W. J., DIECKMANN, M., RHODES, C. & BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**, 237–251.

ROSE, J. A., BERNS, K. I., HOGGAN, M. D. & KOCZOT, F. J. (1969). Evidence for a single-stranded adenovirus-associated genome: formation of a DNA density hybrid on release of viral DNA. *Proceedings of the National Academy of Sciences, U.S.A.* **64**, 863–869.

SALACINSKI, P., HOPE, J., McLEAN, C., CLEMENT-JONES, V., SYKES, J., PRICE, J. & LOWRY, P. J. (1979). A new simple method which allows theoretical incorporation of radio-iodine into proteins and peptides without damage. *Journal of Endocrinology* **81**, 131.

SERJEANT, G. R., MASON, K., TOPLEY, J. M., SERJEANT, B. E., PATTISON, J. R., JONES, S. E. & MOHAMED, R. (1981). Outbreak of aplastic crises in sickle cell anaemia associated with parvovirus-like agent. *Lancet* ii, 595–597.

SHNEERSON, J. M., MORTIMER, P. P. & VANDERVELDE, E. M. (1980). Febrile illness due to a parvovirus. *British Medical Journal* ii, 1580.

SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.

TATTERSALL, P. (1978). Parvovirus protein structure and virion maturation. In *Replication of Mammalian Parvoviruses*, pp. 53–72. Edited by D. C. Ward & P. Tattersall. New York: Cold Spring Harbor Laboratory.

TIJSSEN, P. & KURSTAK, E. (1981). Biochemical, biophysical, and biological properties of densonucleosis virus (parvovirus). III. Common sequences of structural proteins. *Journal of Virology* **37**, 17–23.

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