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A Cytotoxic Nonstructural Protein, NS1, of Human Parvovirus B19 Induces Activation of Interleukin-6 Gene Expression

STANLEY MOFFATT,[1] NOBUYUKI TANAKA,[1] KOHTARO TADA,[1] MASATO NOSE,[2] MASATAKA NAKAMURA,[3] OSAMU MURAOKA,[4] TOSHIO HIRANO,[4] AND KAZUO SUGAMURA[1]*

Departments of Microbiology[1] and Pathology,[2] Tohoku University School of Medicine, Sendai 980-77, Human Gene Sciences Center, Tokyo Medical and Dental University, Tokyo 113,[3] and Department of Molecular Oncology, Biomedical Research Center, Osaka University Medical School, Suita, Osaka 565,[4] Japan

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We examined the biological function of a nonstructural regulatory protein, NS1, of human parvovirus B19. Because of the cytotoxic activity of NS1, human hematopoietic cell lines, K562, Raji, and THP-1, were established as transfectants which produce the viral NS1 protein upon induction by using bacterial lactose repressor/operator system. NS1 was significantly produced in the three transfectant cells in an inducer dose- and time-dependent manner. Surprisingly, these three transfectants secreted an inflammatory cytokine, interleukin-6 (IL-6), in response to induction. However, no production of other related cytokines, IL-1β, IL-8, or tumor necrosis factor alpha, was seen. Moreover, NS1-primed IL-6 induction was transiently demonstrated in primary human endothelial cells. Analysis with luciferase reporter plasmids carrying IL-6 promoter mutant fragments demonstrated that NS1 effect is mediated by a NF-κB binding site in the IL-6 promoter region, strongly implying that NS1 functions as a *trans*-acting transcriptional activator on the IL-6 promoter. Our novel finding, IL-6 induction by NS1, supports the possible relationship between parvovirus B19 infection and polyclonal activation of B cells in rheumatoid arthritis and indicates that NS1 protein may play a significant role in the pathogenesis of some B19-associated diseases by modulating the expression of host cellular genes.

Human parvovirus B19 was discovered in the sera of healthy blood donors in 1975 (8). It is not associated with any diseases; it possesses a linear 5-kb single-stranded DNA as a genome and carries two nonoverlapping reading frames encoding two nonstructural proteins (NS1 and NS2) and two structural proteins (VP1 and VP2). VP1 and VP2 constitute parvovirus particles as minor and major capsid components, respectively, and possess neutralizing epitopes (30, 32, 33, 41). The NS proteins of human parvovirus are essential for viral genome replication (10). NS1 has also been shown to activate transcription from the parvovirus p6 promoter and the human immunodeficiency virus type 1 long terminal repeat (24, 26). Moreover, mouse parvovirus NS1 has the activity of ATP-dependent site-specific endonuclease with DNA helicase, whereas NS2 binds to the 5′ terminus of the viral DNA strand (39). These results are strong indications that NS proteins may play pivotal roles in the replication of the parvovirus genome.

There are several articles reporting the propagation of human parvovirus B19 in vitro with erythroid-lineage cells such as those from bone marrow (28). In particular, we successfully demonstrated the propagation of parvovirus B19 in primary fetal liver cells (40). These cells, however, permit only transient propagation of human parvovirus B19, and no cell line promoting continuous propagation has been established. Accumulating evidence has indicated that NS1 of both mouse and human parvoviruses is toxic to the host cells (4, 27). This may be the primary reason for the lack of parvovirus-producing cell lines, thereby preventing extensive studies not only of parvovirus B19 propagation but also of its pathogenesis.

* Corresponding author. Mailing address: Department of Microbiology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-77, Japan. Phone: 81-22-717-8096. Fax: 81-22-273-2787. Electronic mail address: e21468@cctucc.tohoku.ac.jp.

Since the discovery of parvovirus B19, subsequent efforts have unequivocally shown the involvement of the virus in a myriad of diseases including erythema infectiosum in children, hydrops fetalis, and aplastic crises (5, 21, 42). In addition, the association of parvovirus B19 infection and arthritis has been strongly suggested in several ways (6, 9). Particularly, we previously reported a possible association between parvovirus B19 infection and production of rheumatoid factor (31). Moreover, a supposed parvovirus, RA-1, different from B19, from the synovial tissue of a rheumatoid arthritis patient was reported to be pathogenic in newborn mice (36). Other reports, however, failed to identify the parvovirus B19 antigen or DNA in the synovial fluids of rheumatoid arthritis patients with recent parvovirus B19 infection (6). Abnormal levels of production of inflammatory cytokines, particularly interleukin-6 (IL-6), are closely linked to active rheumatoid arthritis (17), and IL-6 has been implicated in the pathogenesis of autoimmune disease (15). Nevertheless, the link between cytokine production and parvovirus B19 infection has not been established, and there is no evidence to substantiate a direct association of parvovirus B19 with rheumatoid arthritis.

In this study, we investigated whether parvovirus B19 NS1 protein stimulates IL-6 induction, a major manifestation of rheumatoid arthritis, by using cultured mammalian cells stably carrying the NS1 gene. To overcome the problem of parvovirus NS1 cytotoxicity, we adapted an inducible expression system to tightly regulate, on command, the expression of the NS1 protein by using the *Escherichia coli* lactose operon (*lac*) repression mechanism (1, 3, 11). Our results clearly demonstrate that the parvovirus B19 NS1 protein activates IL-6 production, presumably indicating a direct participation of parvovirus B19-associated diseases by modulating the expression of host cellular genes.

MATERIALS AND METHODS

Plasmids. pGEM-1/B19 carrying the whole genome of B19 was a generous gift of J. P. Clewley (Central Public Health Laboratory, London, United Kingdom). The Lac repressor expressing plasmid, p3′SS, and the *lac* operator vector, pOPRSV1CAT, were obtained from Stratagene. The 2.1-kb fragment of the NS1 coding region was prepared by PCR with a set of primers, 5′-CACGAATTCG CGGCCGCTTTTTTGTGAGCTAACTA-3′ and 5′-AATAAGCTTGCGGCC GCTTACACAGCTTAGCAAAT-3′, whose ends contain *Not*I restriction sites and inserted into the *Not*I site of pOPRSV1CAT, generating pOPRSV-NS1. The luciferase reporter plasmids with the wild-type and mutant IL-6 promoter fragments have been described elsewhere (25). The PCR-generated 100-bp fragment from nucleotides 226 to 336, with *Sac*I and *Nhe*I sites, corresponding to the p6 promoter of parvovirus B19 from pGEM-1/B19, was inserted between the *Sac*I and *Nhe*I sites in the luciferase reporter plasmid PGV-B (Toyo Inc., Tokyo, Japan), generating PGV-p6. Another luciferase reporter, pSV2-Luc (containing the heterologous simian virus 40 promoter region) (34), served as a positive control. An expression plasmid for glutathione-*S*-transferase–NS1 fusion protein was constructed by ligation into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia) of the 120-bp fragment amplified by PCR with sense (5′-GAAGGATC CTTTCCATTTAATGATGTA-3′) and antisense (5′-TTCGAATTCATTTTTG ATCTACCCTGGT-3′) primers of the NS1 coding region. A β-galactosidase expression vector, pRSV-βgal, was used as an internal control (34). Sequences of the PCR products and the junction between inserts and vectors were confirmed by DNA sequencing.

Cells. K562 is a human erythroleukemia cell line, Raji is an Epstein-Barr virus transformed B-cell line, and THP-1 is a myelomonocytic cell line. The cells were maintained in RPMI 1640 medium containing 10% fetal calf serum. Primary human umbilical vein endothelial cells (HUVEC), were grown in preformulated medium, EGM-MV, containing bovine brain extract with 3 mg of heparin per ml, 10 μg of human epidermal growth factor per ml, 50 mg of gentamicin per ml, 50 μg of amphotericin B per ml, and 10% fetal calf serum.

Anti-parvovirus NS1 monoclonal antibody. The glutathione-*S*-transferase–NS1 fusion protein with a molecular mass of 32.5 kDa produced from pGEX2T-NS1 in *E. coli* DH5α was purified on a Sepharose 4B column (Pharmacia), and its purity was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. BALB/c mice were subcutaneously immunized with an equal volume of complete Freund adjuvant and boosted with the same antigen twice intraperitoneally. At 24 days after the first immunization, the spleen cells of the immunized mice were fused with SP2/0-Ag 14 myeloma cells and hybridoma cells producing antibodies to NS1 were screened by enzyme-linked immunosorbent assay (ELISA) with the antigens of the purified fusion protein. A representative positive clone was named ParNS1.

Stable transfection. Cells from logarithmically growing cultures were washed with phosphate-buffered saline (PBS) and resuspended at a final concentration of 10^7 cells per ml in PBS. Cell suspensions were chilled on ice, and 10 μg of linearized p3′SS plasmid DNA was added. The cells were then pulsed with a Gene-Pulser electroporator (Bio-Rad), left on ice for 10 min, and cultured at 37°C in 5% CO_2 for 2 days, and then transfectants were selected in medium containing hygromycin B (Sigma) at 250 μg/ml (for K562), 300 μg/ml (Raji), and 300 μg/ml (THP-1). Half the medium was replaced with fresh medium every 2 days. Clones which had stably integrated the *lac* repressor were first checked by reverse transcriptase PCR with total RNA (5 μg) and a pair of primers, 5′-GG ATCCCGCCATGGTATCAACG-3′ and 5′-AGTATCGATCCTGTTAAAT-3′, specific for the *lac* repressor gene and then confirmed by immunoprecipitation with rabbit anti-LacI antiserum (Stratagene). Positive clones were then prepared as above and further transfected by electroporation with 10 μg of pOPRSV-NS1. Transfectants were then selected in medium containing G418 (Sigma) at 550 μg/ml (for K562), 600 μg/ml (Raji), and 600 μg/ml (THP-1), and positive clones were subsequently isolated by immunoprecipitation with anti-NS1 monoclonal antibody, ParNS1.

Luciferase assay. Reporter plasmids were transfected into 5×10^6 cells of K562-NS by the DEAE-dextran method. In brief, cells were washed twice and suspended in 1 ml of RPMI 1640 medium. Plasmid DNA in 1 ml of RPMI 1640 medium containing 250 μg of DEAE-dextran (DEAE-dextran stock solution: 5 mg of DEAE-dextran per ml in 1 M Tris-HCl [pH 7.5]) per ml was mixed with the cell suspension, and cells were incubated for 30 min at 37°C. Na-heparin (1 U/ml in RPMI 1640 medium; Wako, Osaka, Japan) was then added. The cells were cultured for 24 h at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 10 mM, which was determined from a series of initial experiments, was added to the culture for a further 24 h. The cells were lysed in 150 μl of 25% lysis buffer for 10 min at room temperature. Soluble extracts were prepared by centrifugation ($800 \times g$ for 5 min) and assayed for luciferase activity with a PicaGene assay kit (PGK-L100; Toyo Inc.). Light intensity was measured with a luminometer (LB9501; Berthold, Wildbad, Germany). The protein concentration was determined with a protein assay kit (Bio-Rad) and used for normalization of the luciferase assays.

Immunoprecipitation and immunoblotting. Total-cell lysates were obtained from stable NS1 transfectants (5×10^6 cells) with RIPA buffer (pH 7.5) containing 10 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 10 μg of aprotinin per ml. The lysates were then immunoprecipitated with ParNS1 antibody coupled to protein A-Sepharose beads and anti-mouse immunoglobulin G (Zymed). The immunoprecipitates were separated on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride filters (Millipore). After being incubated in PBS containing 3% bovine serum albumin, the blots were probed with ParNS1 and the immune complexes were visualized by enhanced chemiluminescence as described by the manufacturer (Amersham).

Cytokine production. Supernatants were collected from cultures of K562-NS, Raji-NS, THP-1–NS, and transfected HUVEC with or without IPTG treatment. Levels of IL-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) were measured by sandwich ELISA with manufactured kits (Fuji Rebio, Tokyo, Japan).

PCR amplification. PCR was performed with 100 μl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 pmol of a pair of primers, 200 mM deoxynucleoside triphosphates (dNTPs) and 2.5 U of *Pfu* DNA polymerase (Stratagene). A total of 25 cycles of amplification were carried out with experimentally determined optimal temperature cycle parameters for denaturing (94°C for 1 min), annealing (45°C for 2 min), and synthesis (75°C for 3 min) in a DNA thermal cycler (Perkin-Elmer). The PCR products were then examined on a 7% polyacrylamide gel by visualization with ethidium bromide.

RESULTS

Hematopoietic cell lines inducibly producing expressing NS1. Previous studies indicated that no cell line which permanently produces the parvovirus B19 NS1 protein is readily available because of the lethal effects of this protein on cells. We thus attempted to establish cell lines which express NS1 upon induction to facilitate investigation into the biological function of NS1. Since parvovirus B19 has been shown to infect erythroid lineage cells per se, the hematopoietic cell lines K562, Raji, and THP-1 were used for this purpose. These cells were first transfected with plasmid p3′SS carrying the bacterial *lac* repressor gene, and the transfected cells K562-Lac, Raji-Lac, and THP-1–Lac were confirmed to have integrated the *lac* repressor in both the cytoplasmic and nuclear fractions (Fig. 1A) by probing with anti-*lac* repressor antiserum (Stratagene). *lacI* expression was observed to be unequal in the two cellular compartments, agreeing with previous reports that LacI protein is localized predominantly in the cytoplasm, a fraction of which migrates to the nucleus to bind DNA. The transfectants K562-Lac, Raji-Lac, and THP-1–Lac were further transfected with an NS1 expression plasmid (pOPRSV-NS1) in which the NS1 coding region is transcriptionally controlled by the *lac* repressor-operator system, generating K562-NS, Raji-NS, and THP-1-NS, respectively. The doubly transfected cell lines were examined for their abilities to produce the NS1 protein upon induction with IPTG, which is an inhibitor of the *lac* repressor, and clones exhibiting stringent regulation of NS1 expression were selected.

To specifically assess NS1 expression dynamics, we raised a new monoclonal antibody (ParNS1) specific to the parvovirus B19 NS1 protein but not to NS2. This antibody was raised by immunizing mice with bacterially produced glutathione-*S*-transferase–NS1 fusion protein and tested by Western blot (immunoblot) analysis and immunoprecipitation. After cell culture under indicated conditions, K562-NS, Raji-NS, and THP-1–NS were lysed and NS1 was detected by a combination of immunoprecipitation and Western blotting with ParNS1. Appreciable NS1 protein could not be seen with the cells cultured without IPTG (Fig. 1B). When the cells were cultured with IPTG, however, NS1 became detectable in the three cell lines in a dose-dependent manner. A concentration of 2 mM IPTG induced NS1 production, which was maximal at 10 to 16 mM IPTG in K562-NS and Raji-NS whereas THP-1–NS cells required 8 mM IPTG for minimal induction. These results demonstrated that the *lac* repressor-operator strictly regulates the expression of parvovirus B19 NS1 in hematopoietic cell lines.

To evaluate the kinetics of NS1 induction, the rate of ap-

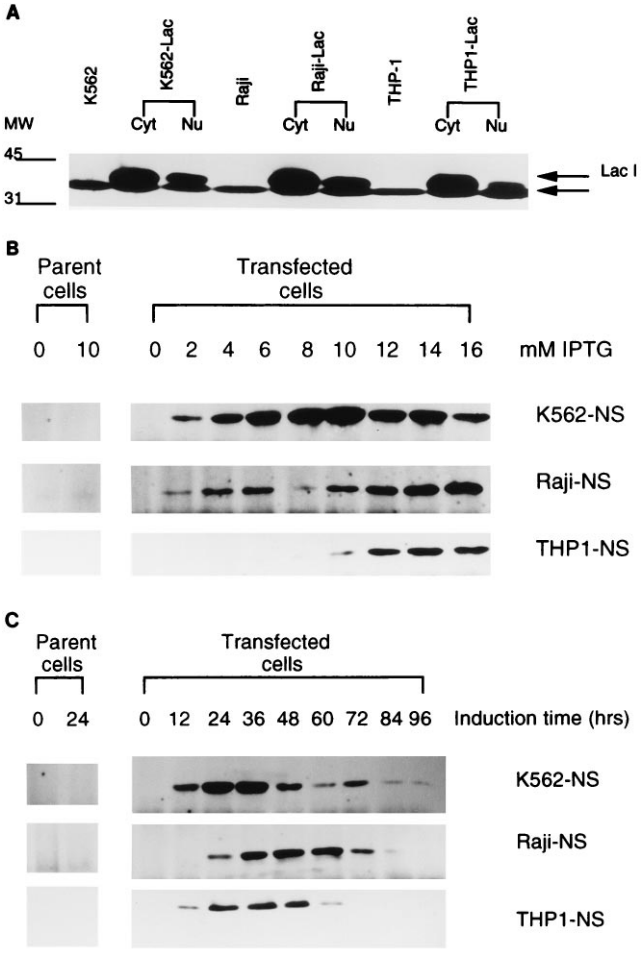

FIG. 1. Establishment of NS1-expressing cells. (A) Immunoprecipitation of the Lac repressor protein. The cytoplasmic (Cyt) and nuclear (Nu) extracts from K562-Lac, Raji-Lac and THP-1–Lac transfectants and the total cell lysates of their respective parental cells were analyzed for the expression of the *lac* repressor protein by probing the blot with a LacI antiserum. LacI migrated as an almost 38.8-kDa protein but was absent in the parental cells. MW indicates molecular mass in kilodaltons. The arrow under that of LacI indicates a nonspecific band. (B) Inducible expression of NS1. NS1 is produced in an IPTG dose-dependent manner. K562-NS, Raji-NS and THP-1–NS cells (5×10^6 cells) were cultured with or without the indicated concentrations of IPTG for 24 h. Cell lysates were immunoprecipitated with ParNS1 monoclonal antibody. The immunoprecipitates were applied to SDS-polyacrylamide gel electrophoresis with a 10% gel and then blotted with filters, which were visualized by the enhanced chemiluminescence detection system after probing with the same antibody. Parent cells represent untransfected K562, Raji, and THP-1 cells. They were similarly cultured with (10 mM) or without IPTG, immunoprecipitated, and immunoblotted. The NS1 protein detected was estimated to be approximately 71 kDa. (C) Kinetics of NS1 induction in transfected cells. K562-NS, Raji-NS, and THP-1–NS were cultured in the presence of 10 mM IPTG for the indicated periods. Immunoprecipitation and immunoblotting were carried out as described in the legend to panel A. Parent cells (K562, Raji, and THP-1) were equally cultured with (10 mM) or without IPTG for 24 h.

pearance and disappearance of NS1 protein in cells following addition of 10 mM IPTG to the inducible cultures was measured over several days. NS1 protein was detected 12 h after exposure of K562-NS and THP-1–NS to IPTG and 24 h after exposure of Raji-NS, with the level becoming maximal at 24 to 36 h for K562-NS, 24 to 48 h for THP-1–NS, and 36 to 60 h for Raji-NS (Fig. 1C). This expression profile was followed by a differential decline while in the presence of optimum amounts of the inducer IPTG, indicating that the induced NS1 expression is transient.

TABLE 1. Dose-dependent induction of IL-6 in NS1 transfectant cells[a]

Cell line	IPTG concn (mM)	Concn of induced IL-6 (pg/ml)
K562-NS	0	8
	10	125
Raji-NS	0	18
	10	235
THP-1–NS	0	10
	10	210

[a] K562-NS, Raji-NS, and THP-1–NS cells were cultured without or with 10 mM IPTG for 24 h. Culture supernatants were recovered for the IL-6 assay by sandwich ELISA. Values are the mean concentrations derived from duplicate experiments.

NS1-dependent induction of IL-6 production. With NS1-transfected cell lines, we examined the effects of NS1 on the production of inflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α, which have been implicated in the pathogenesis of rheumatoid arthritis (6, 7, 15, 17, 38). During cultures with IPTG, culture supernatants were collected and tested for the production of these cytokines by ELISA. Little or no production of IL-1β, IL-8, or TNF-α was seen with the three cell lines for 60 h after addition of 10 mM IPTG (data not shown). In contrast, the three cell lines (K562-NS, Raji-NS, and THP-1–NS) secreted significant amounts of IL-6 into the culture supernatants in response to the addition of 10 mM IPTG. These data demonstrated that parvovirus B19 NS1 induces the production of IL-6. Upon induction, parental and *lac* repressor-transfected cells did not show any appreciable change in the production of IL-6 from background levels, which are similar to those of the NS1-transfected cells without IPTG (Table 1; data not shown). We then designed an experiment to see the kinetics of IL-6 secretion upon NS1 induction. As illustrated in Fig. 2, high levels of IL-6 secretion were observed at 12 h after addition of 10 mM IPTG, roughly paralleling the induction of

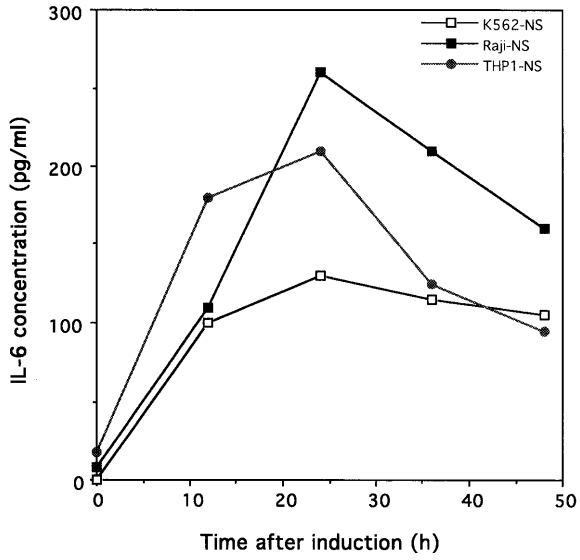

FIG. 2. IL-6 levels in culture supernatants of NS1-expressing cells. K562-NS, Raji-NS, and THP-1–NS were cultured with 10 mM IPTG for the indicated periods. IL-6 levels in culture supernatants were determined by sandwich ELISA. The data represent the mean of duplicate experiments, and the absolute values were obtained after subtracting the corresponding values for the zero standards.

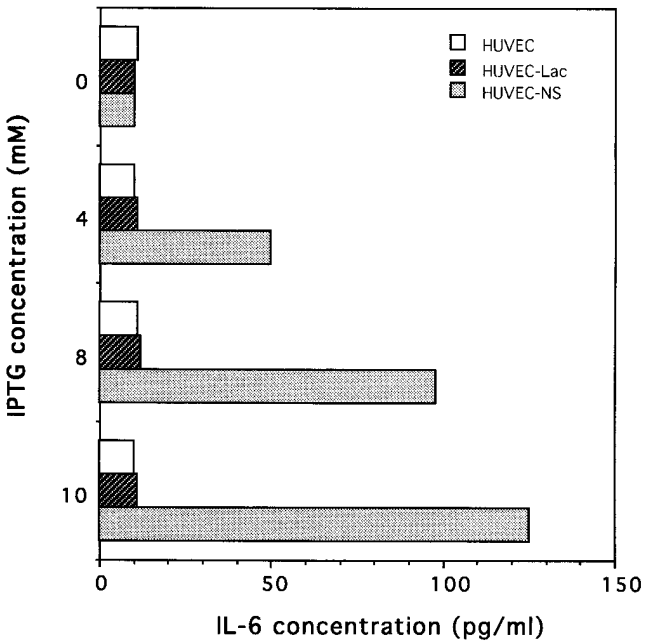

FIG. 3. NS1-dependent IL-6 production in primary endothelial cells. HUVEC were transiently transfected with 10 μg each of p3′SS and pOPRSV-NS1 and then cultured with the indicated concentrations of IPTG for 24 h. Culture supernatants were harvested and then assayed for IL-6 concentration by sandwich ELISA. Three independent experiments gave similar results.

NS1. The secretion reached a peak at 24 h and was followed by a decline.

NS1 effects on IL-6 secretion in primary endothelial cells. To investigate whether IL-6 induction by parvovirus B19 NS1 was restricted to hematopoietic cells, we sought to examine NS1-primed IL-6 induction in primary HUVEC. HUVEC were transiently introduced with p3′SS and pOPRSV-NS1 simultaneously, after which IL-6 production upon treatment with different IPTG concentrations was measured. IL-6 concentrations in the culture supernatants of transfected HUVEC increased depending on the doses of IPTG, similar to the hematopoietic cell lines. No increase in IL-6 concentrations in response to IPTG was observed with parental HUVEC or HUVEC transfected with only the repressor (Fig. 3). These results illustrated that parvovirus B19 NS1 can transactivate the expression of the IL-6 gene in cells of the vascular system.

Transactivation of the IL-6 promoter by NS1. To gain insight into the molecular mechanism of IL-6 induction, we examined the effects of NS1 on the IL-6 promoter in transient transfection. A luciferase reporter plasmid (pIL6-luc) (25) carrying the IL-6 promoter region was transiently transfected into K562-NS cells by the DEAE-dextran method. The cells were then cultured with either 5 or 10 mM IPTG for 24 h and examined for luciferase activity. After correction of the transfection efficiency by the β-galactosidase activity which resulted from the cotransfection of plasmid RSV-βgal used as an internal control, the luciferase activities were expressed as ratios (fold induction) to those from uninduced cells. Additions of IPTG at 5 and 10 mM resulted in profound increases in luciferase activities, with 3- and 5.5-fold induction, respectively (Fig. 4). Parental K562 or K562-Lac failed to induce IL-6 promoter activity significantly even after treatment with IPTG. These results demonstrated that the IL-6 promoter is *trans* activated by parvovirus B19 NS1. Similar increases in luciferase activity were seen with the parvovirus p6 promoter in our NS1 induction system, confirming the previous study that NS1 protein is essential for its own viral gene expression.

NF-κB element responsible for NS1-induced *trans*-activation. We further wished to explore the elements in the IL-6 promoter responsible for NS1-dependent IL-6 induction. To characterize such an element(s), we used a series of 5′ deletion mutants of the IL-6 promoter region from bp 592 to 36 upstream of the IL-6 transcription start site, generating pIL6-592 to pIL6-36 by insertion into a luciferase reporter plasmid (PGV-B). The luciferase activities in K562-NS cells transfected with pIL6-592, pIL6-224, and pIL6-181 were as high as that with the wild-type IL-6 promoter (pIL-6) when cells were cultured with 10 mM IPTG (Fig. 5). Fragments deleted up to nucleotide positions −138 and −108 reproducibly showed low luciferase activities, with inductions of 1.8- and 1.7-fold, respectively. Plasmid pIL6-73 exhibited higher luciferase activity, closer to that of pIL6-592, pIL6-224, and pIL6-181 than to that of pIL6-138 and pIL6-108. The other mutant fragment carrying up to nucleotide position −36 (pIL6-36) showed luciferase activity indistinguishable from the background level in the absence of IPTG. These data suggest that there are at least two positive regulatory regions (−181 to −138 and −73 to −36) and a negative regulatory region (−108 to −73) in NS1-mediated IL-6 induction.

It is worth noting that the putative NS1 responsive region between −73 and −36 includes the NF-κB (IL-6κB)-binding site. We thus addressed the question whether the NF-κB-binding site functions in NS1-dependent activation of the IL-6 gene promoter. A mutant fragment with a substitution of GGGATT to CTCGAG in the NF-κB-binding site was inserted into the reporter plasmid, and the resultant plasmid (pIL6-mtκB181)

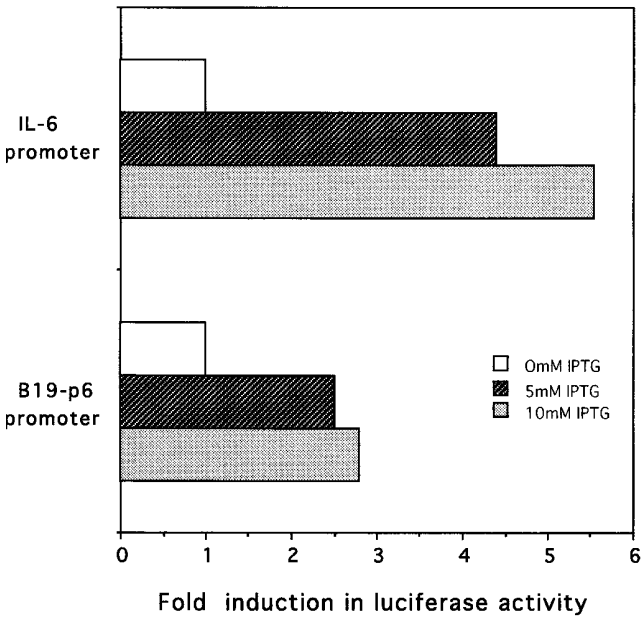

FIG. 4. Activation of the IL-6 promoter by NS1. K562-NS cells were transiently transfected with 5 μg of luciferase reporter plasmids with wild-type and mutant IL-6 promoter sequences by the DEAE-dextran method. The cells were cultured without (0 mM) or with 5 or 10 mM IPTG for 24 h, and luciferase activities were determined. The activities were normalized by protein concentration and transfection efficiency. Results are expressed as fold inductions, which are ratios of normalized luciferase activities of induced cells to those of uninduced cells. Respective mean results of duplicate experiments are represented.

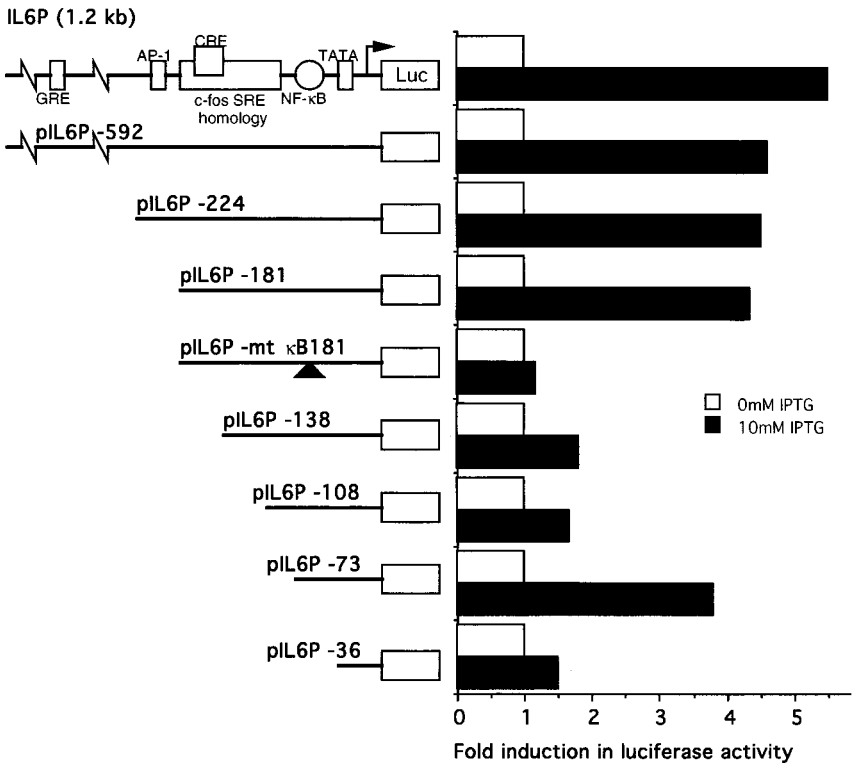

FIG. 5. NS1-responsive region of the IL-6 promoter. Luciferase reporter plasmids bearing wild-type or IL-6 promoter mutants were transfected into K562-NS cells by the DEAE-dextran method. Cells were cultured with or without 10 mM IPTG for 24 h, and luciferase activities in cell lysates were determined as described in Materials and Methods. The results of two independent experiments are expressed as fold inductions, as in Fig. 4. Relevant transcription factor-binding sites are also indicated. Numbers indicate 5′-far-end nucleotides upstream of the IL-6 transcription start site. All constructs contain the native IL-6 TATA box and initiation sites. The solid triangle under pIL6P-mtκB181 indicates a change of the nucleotide sequence GGGATT to CTCGAG.

was introduced into K562-NS transiently to measure the luciferase activity after induction of NS1. The mutant fragment gave little, if any, increase in luciferase activity in K562-NS cells treated with 10 mM IPTG, while the −181 fragment containing the wild-type NF-κB site showed an approximately 4.5-fold increase upon induction (Fig. 5). This contrast clearly indicates that the NF-κB (IL-6κB) site is critical for induction of the IL-6 gene promoter by NS1.

DISCUSSION

In this study, by using the bacterial *lac* repressor-operator system, we have demonstrated the feasibility of inducible production of human parvovirus B19 NS1 protein. To our knowledge, this is the first successful stringent regulation of this cytotoxic viral gene product. Consequently, we shed light on a hitherto unknown biochemical function of NS1 and its implication in parvovirus B19-infected disease pathogenesis.

NS1 production controlled by the *lac* repressor-operator system is dependent on the dose of IPTG. We observed that addition of IPTG to the medium did not cause any appreciable changes in cell morphology and viability. Our results demonstrated that, unexpectedly, the IPTG-dependent NS1 production is transient. We do not know why NS1 production ceases even in the presence of adequate amounts of IPTG in the medium. However, we believe that this is a consequence of the cytotoxic effects of the NS1 protein. Alternatively, the production of NS1 may induce a mechanism by which NS1 itself is degraded. Nevertheless, an advantage of this inducible production is the wide selection of cells which may produce NS1. Indeed, we succeeded in induction of NS1 expression in primary culture cells of umbilical vein endothelium.

Our study with the induction system provides evidence that human parvovirus B19 induces IL-6 production. The NS1-dependent IL-6 production is not restricted to erythroid lineage cells, which have been shown to be susceptible to parvovirus B19. In addition to lymphoid and monocytic lineage cell lines, primary endothelial cells produced IL-6 in response to NS1. It is therefore likely that a wide variety of cells would secrete IL-6 when expressing the NS1 protein. The amounts of IL-6 produced in the doubly-transfected cells, however, are not necessarily proportional to the level of NS1 expression, and the kinetics of IL-6 production do not parallel the kinetics of NS1 production. These results indicate that IL-6 production is dependent not only on NS1 but also on the intracellular environment peculiar to each cell line.

The transient-transfection assay with deletion and substitution mutants of the IL-6 promoter revealed that the NF-κB site is primarily responsible for NS1-dependent induction of IL-6. NF-κB may be activated by NS1 via a direct pathway. This may be postulated since NS1 is a *trans*-acting transcriptional activator of its own viral promoter, p6. In addition, this assumption is reminiscent of a human retrovirus nonstructural regulatory factor, human T-cell leukemia virus type 1 (HTLV-1) Tax. The Tax regulatory factor necessary for the propagation of the virus induces the activation of IL-6 gene expression, presumably through the NF-κB-binding site in the IL-6 gene promoter (14, 25). HTLV-1 has also been shown to activate other cellular genes such as those for IL-2, IL-2 receptor α chain, c-*fos*, TNF-β, and a host of others (37). However, NS1 did not

enhance the expression of these genes in luciferase reporter assays with K562-NS, Raji-NS, and THP-1–NS cells (data not shown). The lack of effect of NS1 on the IL-2 gene promoter, which also carries an NF-κB site, implies that NF-κB alone is not sufficient for activation of the IL-2 gene promoter and possibly other NF-κB-containing promoters. The effect of NS1 on the NF-κB site may thus be determined by the context of enhancer elements in promoters.

It is of interest that another human retrovirus, human immunodeficiency virus type 1 has a *trans*-acting factor, Tat, which induces the production of IL-6 (12). Similar to HTLV-1 Tax and human immunodeficiency virus type 1 Tat, parvovirus NS1 has been characterized to be necessary for viral propagation, presumably playing an essential role in viral genome replication and/or the regulation of expression of viral genes. Furthermore, parvovirus B19 NS1 resembles HTLV-1 Tax and human immunodeficiency virus type 1 Tat in that all three may be directly implicated in pathogenesis by the mechanisms previously proposed (15). In particular, HTLV-1 transgenic mice manifested inflammatory arthropathy resembling rheumatoid arthritis (13, 19). Judging from the close association of parvovirus B19 infection and inflammatory arthritis coupled with NS1-mediated IL-6 induction, the physiological mechanism underlying the manifestation of rheumatoid arthritis due to a trigger by the two viral infections probably involves similar immunological pathways.

In view of the tremendous amount of inflammatory cytokines, particularly IL-6, in fluids from inflamed joints of patients with rheumatoid arthritis, it is important to know that administration of anti-IL-6 antibody is associated with complete inhibition of major manifestations of rheumatoid arthritis, particularly acute-phase proteins, and this is reflected by a substantial decrease in IL-6 production (2). The implication of IL-6 in autoimmune diseases is further authenticated in patients with cardiac myxoma (18) and Castleman's disease (22), in whom the autoimmune symptoms and autoantibody production disappeared either after the surgical removal of the myxoma cells or after the resection of the hyperplastic lymph node, resulting in dramatic reduction of IL-6 production (15). Deregulated expression of IL-6 has also been strongly suggested to be involved in the pathogenesis of polyclonal B-cell activation (16) and synovial cell proliferation in the synovium of rheumatoid arthritis patients (20, 23). These observations strongly suggest that abnormal expression of IL-6 may contribute, presumably as a result of enhancement of production of autoantibody including rheumatoid factor, to the generalized autoimmune diseases such as rheumatoid arthritis. NS1-primed IL-6 induction is thus important and interesting in the context of B19-associated rheumatoid arthritis. Our results are also consistent with recent reports which have ascribed the abnormal release of IL-6 to the priming of antigens (12, 29, 35).

The present data suggest the possibility that cells which are nonpermissive to parvovirus B19 infection are led, upon producing NS1, to the stimulation of endogenous IL-6 gene expression. This finding strongly implicates NS1 as a pivotal gene participating in the pathogenesis of parvovirus B19-associated diseases. Taken together, our results would be a valuable paradigm for further studies on the pathogenesis of parvovirus B19-associated diseases, in particular rheumatoid arthritis.

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REFERENCES

1. **Baim, S. B., M. A. Labow, A. J. Levine, and T. Shenk.** 1991. A chimeric mammalian transactivator based on the lac repressor that is regulated by temperature and isopropyl B-D-thiogalactopyranoside. Proc. Natl. Acad. Sci. USA **88:**5072–5076.
2. **Bataille, R., B. Barlogie, Z. Y. Lu, J. F. Rossi, T. Lavabre-Bertrand, T. Beck, J. Wijdeness, J. Brochier, and B. Klein.** 1995. Biologic effects of anti-interleukin-6 murine monoclonal antibody in advanced multiple myeloma. Blood **86:**685–691.
3. **Brown, M., J. Figge, U. Hansen, C. Wright, K.-T. Jeang, G. Khoury, D. M. Livingston, and T. M. Roberts.** 1987. lac repressor can regulate expression from a hybrid SV40 early promoter containing a lac operator in animal cells. Cell **49:**603–612.
4. **Caillet-Fauquet, P., M. Perros, A. Brandenburger, P. Spegelaere, and J. Rommelaere.** 1990. Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. EMBO J. **9:**2989–2995.
5. **Cassinotti, P., M. Weitz, and G. Siegl.** 1993. Human parvovirus B19 infection: routine diagnosis by a new nested polymerase chain reaction assay. J. Med. Virol. **40:**228–234.
6. **Cohen, B. J., M. M. Buckley, J. P. Clewley, V. E. Jones, A. H. Puttick, and R. K. Jacoby.** 1986. Human parvovirus infection in early rheumatoid and inflammatory arthritis. Ann. Rheum. Dis. **45:**832–838.
7. **Cope, A. P., D. Aderka, M. Doherty, H. Engelmann, D. Gibbons, A. C. Jones, F. M. Brennan, R. N. Maini, D. Wallach, and M. Feldmann.** 1992. Increased levels of soluble tumor necrosis factor receptors in the sera and synovial fluid of patients with rheumatic diseases. Arthritis Rheum. **35:**1160–1169.
8. **Cossart, Y. E., A. M. Field, B. Cant, and D. Widdows.** 1975. Parvovirus-like particles in human sera. Lancet **i:**72–73.
9. **Dijkmans, B. A. C., A. M. W. van Elsacker-Niele, M. M. M. Salimans, G. A. van Albada-Knipers, E. de Vries, and H. T. Weiland.** 1988. Human parvovirus B19 DNA in synovial fluid. Arthritis Rheum. **31:**279–281.
10. **Doerig, C., B. Hirt, J. Antonietti, and P. Beard.** 1990. Nonstructural proteins of parvovirus B19 and minute virus of mice control transcription. J. Virol. **64:**387–396.
11. **Figge, J., C. Wright, C. J. Collins, M. T. Roberts, and M. D. Livingstone.** 1988. Stringent regulation of stably integrated chloramphenicol acetyl transferase genes by E. coli lac repressor in mammalian cells. Cell **52:**713–722.
12. **Giuseppe, S., M. R. Ruocco, C. Ambrosino, M. Mallardo, V. Giordano, F. Baldassarre, E. Dragonetti, I. Quinto, and S. Venuta.** 1994. The expression of the interleukin 6 gene is induced by the human immunodeficiency virus 1 TAT Protein. J. Exp. Med. **179:**961–971.
13. **Green, E. J., S. H. Hinrichs, J. Vogel, and G. Jay.** 1989. Exocrinopathy resembling Sjoegren's syndrome in HTLV-1 tax transgenic mice. Nature (London) **341:**72–74.
14. **Hall, W. H.** 1994. Human T-cell lymphotropic virus type 1 and cutaneous T cell leukemia/lymphoma. J. Exp. Med. **180:**1581–1585.
15. **Hirano, T.** 1992. Interleukin 6 and autoimmunity and plasma cell neoplasis. Res. Immunol. **143:**759–763.
16. **Hirano, T., S. Akira, T. Taga, and T. Kishimoto.** 1990. Biological and clinical aspects of interleukin 6. Immunol. Today **11:**443–449.
17. **Hirano, T., T. Matsuda, M. Turner, N. Miyasaka, G. Buchan, B. Tang, K. Sato, M. Shimizu, R. Maini, M. Feldman, and T. Kishimoto.** 1988. Excessive production of interleukin-6/B cell stimulatory factor-2 in rheumatoid arthritis. Eur. J. Immunol. **18:**1797–1801.
18. **Hirano, T., T. Taga, K. Yasukawa, K. Nakajima, N. Nakano, F. Takatsuki, M. Shimizu, A. Murashima, S. Tsunasawa, F. Sakayama, and T. Kishimoto.** 1987. Human B cell differentiation factor defined by an anti-peptide antibody and its possible role in autoantibody production. Proc. Natl. Acad. Sci. USA **84:**228–231.
19. **Iwakura, Y., M. Tosu, E. Yoshida, M. Takiguchi, K. Sato, I. Kitajima, K. Nishioka, K. Yamamoto, T. Takeda, M. Hatanaka, H. Yamamoto, and T. Sekiguchi.** 1991. Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-1. Science **253:**1026–1028.
20. **Jorgensen, C., J. Angel, and C. Fournier.** 1991. Regulation of synovial cell proliferation and prostaglandin E2 production by combined action of cytokines. Eur. Cytokine Netw. **2:**207–215.
21. **Kurtzman, G., N. Frickhofen, J. Kimball, D. W. Jenkins, A. W. Nienhuis, and N. S. Young.** 1989. Pure red-cell aplasia of 10 years duration due to persistent parvovirus B19 infection and its cure with immunoglobulin therapy. N. Engl. J. Med. **321:**519–523.
22. **Leger-Ravet, M. B., M. Peuchmaur, O. Devergne, J. Aoudouin, M. Raphael, J. Van Damme, P. Galanaud, J. Diebold, and D. Emilie.** 1991. Interleukin-6 gene expression in Castleman's disease. Blood **78:**2923–2930.
23. **Mihara, M., Y. Moriya, T. Kishimoto, and Y. Ohsugi.** 1995. Interleukin-6 (IL-6) induces the proliferation of synovial fibroblastic cells in the presence of soluble IL-6 receptor. Br. J. Rheum. **34:**321–325.
24. **Mikio, M., S. Wong, M. Kawase, N. S. Young, and S. Kajigaya.** 1994. A putative nucleoside triphosphate-binding domain in the nonstructural protein of B19 parvovirus is required for cytotoxicity. J. Virol. **68:**8443–8446.
25. **Muraoka, O., K. Tsuneyasu, T. Makoto, and T. Hirano.** 1993. Transcriptional activation of the interleukin-6 gene by HTLV-1 p40tax through an

NF-κB-like binding site. Immunol. Lett. **37:**159–165.
26. **Nathalie, S., M. Frederick, A. Marc, and H. Uriel.** 1993. Transactivation of the long-terminal repeat of human immunodeficiency virus type 1 by the parvovirus B19 NS1 gene product. J. Gen. Virol. **74:**2011–2014.
27. **Ozawa, K., J. Ayub, S. Kajigaya, T. Shimada, and N. S. Young.** 1988. The gene encoding the non-structural protein of B19 (human) parvovirus may be lethal in transfected cells. J. Virol. **62:**2884–2889.
28. **Ozawa, K., G. Kurtzman, and N. Young.** 1986. Replication of the B19 parvovirus in human bone marrow cultures. Science **233:**883–886.
29. **Peebles, R. S., C. R. Maliszewski, T. A. Sato, J. Hanley-Hyde, I. G. Maroulakou, R. Hunziker, J. P. Schneck, and J. E. Green.** 1995. Abnormal B-cell function in HTLV-1 tax transgenic mice. Oncogene **10:**1045–1051.
30. **Saikawa, T., S. Anderson, M. Mimoeda, S. Kajigaya, and N. S. Young.** 1993. Neutralizing epitope of B19 parvovirus cluster in the VP1 and VP1-VP2 junction regions. J. Virol. **67:**3004–3009.
31. **Sasaki, T., Y. Takahashi, K. Yoshinaga, and K. Sugamura.** 1989. An association between human parvovirus B19 infection and autoantibody production. J. Rheumatol. **16:**708–709.
32. **Sato, H., J. Hirata, N. Furukawa, H. Kuroda, Y. Shiraki, Y. Maeda, and K. Okochi.** 1991. Identification of the region including the epitope for a monoclonal antibody which can neutralize human parvovirus B19. J. Virol. **65:** 5845–5849.
33. **Sato, H., J. Hirata, H. Kuroda, Y. Shiraki, Y. Maeda, and K. Okochi.** 1991. Identification and mapping of neutralizing epitopes of human parvovirus B19 by using human antibodies. J. Virol. **65:**5845–5849.
34. **Shibata, K., Y. Muraosa, Y. Tomota, H. Tagami, and S. Shibahara.** 1992. Identification of a cis-acting element that enhances the pigment cell-specific expression of the human tyrosinase gene. J. Biol. Chem. **267:**20584–20588.
35. **Shibata, S., T. Sasaki, A. Hatakeyama, Y. Munakata, Y. Hirabayashi, and K. Yoshinaga.** 1992. The clonal frequency analysis of B-cells producing pathogenic anti-DNA antibody-associated idiotypes in systemic lupus erythematosus. Clin. Immunol. Immunopathol. **63:**252–258.
36. **Simpson, R. W., L. McGinty, L. Simon, C. A. Smith, C. W. Godzeski, and R. J. Boyd.** 1984. Association of parvoviruses with rheumatoid arthritis of humans. Science **233:**1425–1428.
37. **Sugamura, K., and Y. Hinuma.** 1993. Human retroviruses: HTLV-1 and HTLV-II, p. 399–435. *In* J. A. Levy (ed), Retroviradae, vol. 2. Plenum Press, New York.
38. **Thompson, R. C., D. J. Dripps, and S. P. Eisenberg.** 1992. Interleukin-1 receptor antagonist (IL-1ra) as a probe and as a treatment for IL-1 mediated disease. Int. J. Immunopharmacol. **14:**475–480.
39. **Wilson, G. M., H. K. Gindal, D. E. Yeung, W. Chen, and C. R. Astell.** 1991. Expression of minute virus of mice non-structural protein in insect cells: purification and identification of ATPase and helicase activities. Virology **185:**90–98.
40. **Yaegashi, N., H. Shiraishi, T. Takeshta, M. Nakamura, and K. Sugamura.** 1989. Propagation of human parvovirus B19 in primary culture of erythroid lineage cells derived from fetal liver. J. Virol. **63:**2422–2426.
41. **Yoshimoto, K., S. Rosenfeld, N. Frickhofen, D. Kennedy, S. Kajigaya, and N. S. Young.** 1991. A second neutralizing epitope of B19 parvovirus implicates the spike region in the immune response. J. Virol. **65:**7056–7060.
42. **Young, N. S.** 1993. B19 parvovirus, p. 75–117. *In* N. S. Young (ed), Viruses and bone marrow. Marcel Dekker, Inc., New York.