INFECTION AND IMMUNITY, Nov. 1992, p. 4872–4880
0019-9567/92/114872-09$02.00/0

Vol. 60, No. 11

Electrophoretic Karyotypes of Clinical Isolates of *Coccidioides immitis*

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Received 28 April 1992/Accepted 1 September 1992

Chromosomes of the fungal respiratory pathogen, *Coccidioides immitis*, were separated by contour-clamped homogeneous electric field gel electrophoresis. Twelve isolates were examined, the majority of which showed four chromosomes with a range of molecular size from 11.5 to 3.2 Mb. Three isolates (C634, C735, and L) revealed three chromosomal bands under the conditions employed for electrophoretic separation. However, in two of these isolates (C634 and C735), four chromosomes were visible on membrane transfers of pulsed-field gels after Southern hybridization between the chromosomal DNA and selected DNA probes. The probes included a conserved ribosomal gene and three previously described cDNAs isolated from *C. immitis* expression libraries. The L isolate was determined to have the same genome size as a typical four-chromosome isolate on the basis of microspectrophotometric comparison of fluorescence intensity of the ethidium bromide-stained nuclear DNA. The genome size of *C. immitis* determined by microspectrophotometry was approximately 28.2 ± 2.6 Mb. The calculated genome size based on addition of the average molecular weights of chromosomal bands separated by contour-clamped homogeneous electric field gel electrophoresis was approximately equal to the estimate derived from the spectrophotometric analyses. This is the first report of the electrophoretic karyotype of *C. immitis*.

Coccidioides immitis is a respiratory fungal pathogen of humans which is characterized by a desert soil-inhabiting saprobic phase and a morphogenetically unique parasitic phase (11). Arthroconidia produced by the soil-borne mycelia are the infectious propagules of *C. immitis* (9). If inhaled, each viable conidium can differentiate into a large coenocytic spherule, the contents of which convert into a multitude of tiny endospores (11). The endospores function in dissemination of the pathogen from sites of infection within the lungs. *C. immitis* has no recognized sexual phase, or teleomorph (19), and the respiratory pathogen is classified as a member of the Fungi Imperfecti (17). Currah (14), however, has suggested that *C. immitis* is phylogenetically related to the ascomycetous fungi and has included the monospecific genus in the family *Onygenaceae*, a family within the division *Ascomycota*. An important piece of evidence which supports this taxonomic alignment of *C. immitis* has been derived from results of developmental and ultrastructural studies of spherule-endospore formation. Electron-microscopic examinations of stages of spherule segmentation have revealed that this process occurs by septal formation, which is comparable to the mechanism of septation in ascomycetous fungi (11, 31, 33). The events which lead to endospore formation in *C. immitis* are clearly distinct from sporangial cleavage and sporangiospore formation in the class *Zygomycetes* (2). *C. immitis* was originally, but erroneously, considered a member of this latter group of fungi (6, 15, 37). Additional evidence for taxonomic alignment of *C. immitis* with the *Ascomycetes* has been derived from results of ecological, cytological, and biochemical comparisons (11, 22, 32, 34).

Early cytological studies of *C. immitis* focused on nuclear cycles of the saprobic and parasitic phases and made use of basic fuchsin- and aceto-orcein-staining techniques for light-microscopic examinations (22, 34). Results of the first karyological study of *C. immitis* (22) indicated that the chromosome number for the pathogen was three, while in a later report the investigators suggested that four pairs of chromosomes were present (34). The advent of pulsed-field gel electrophoresis (30) has made it possible to resolve such controversies over chromosome number and simultaneously provide valuable information on genome size and gene linkage, which has facilitated construction of physical maps (1, 23, 25, 27). Results of comparative pulsed-field and other DNA electrophoretic separation studies have provided the basis for identification of taxon-specific fungal genes and their subsequent use in evaluation of potential anamorph-teleomorph connections, examinations of fungal phylogeny, and resolution of other taxonomic problems (35). In this paper, we examine the karyotypes of 12 clinical isolates of *C. immitis* by contour-clamped homogeneous electric field (CHEF) gel electrophoresis (5). DNA probes derived from a conserved ribosomal gene fragment and three previously cloned *C. immitis* genes (12, 20, 21) were used in hybridization experiments to confirm the number of chromosomal bands and compare gene linkage patterns of the different isolates. Results of CHEF analyses were correlated with data obtained from cytofluorometric determinations of total nuclear DNA content, and together these techniques were used to estimate the genome size of *C. immitis*. This is the first report of an electrophoretic karyotype analysis of *C. immitis*.

MATERIALS AND METHODS

Isolates examined. A total of 12 clinical isolates of *C. immitis* were examined. Seven isolates were provided by S. H. Sun (Veterans Administration Hospital, San Antonio, Tex.), deposited as University of Texas (UT) cultures, and assigned accession numbers (UT 17 to 19, 22, and 24 to 26). Also included were two previously reported (21) clinical isolates (C634 and C735), the Silveira strain (from S. H. Sun) (34), a clinical isolate (L) provided by D. Pappagianis (Uni-

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versity of California at Davis), and an isolate (C24) obtained from the American Type Culture Collection (ATCC 34615; Rockville, Md.). A chromosomal size standard preparation (fragment sizes of 3.5, 4.6, and 5.7 Mb) derived from *Schizosaccharomyces pombe* was purchased from Bio-Rad Laboratories (Richmond, Calif.). An isolate of *Neurospora crassa*, originally obtained from the Fungal Genetic Stock Center (no. 339; Kansas City, Kans.), was provided to us by A. Riggs (University of Texas at Austin). This strain was used for preparation of chromosomal DNA, which was also employed as a size standard (26).

Preparation of chromosomal DNA samples. Two methods of preparation of intact chromosomal DNA, which are modifications of the (i) liquid spheroplast procedure and (ii) agarose spheroplast method of Orbach et al. (26), were employed. In the liquid spheroplast procedure, the mycelial phase of *C. immitis* was grown in liquid medium which contained 1% glucose plus 0.5% yeast extract (GYE) at 30°C for 48 h in a gyratory shaker-incubator. The mycelial mat of *C. immitis* was isolated by filtration (approximately 1 g [wet wt]) and washed three times with 50 mM EDTA (pH 8.0). The mycelial fraction was resuspended in 10 ml of 50 mM sodium citrate buffer (pH 5.7) containing 1 M sorbitol and 50 mM EDTA. To this mixture, 10 μl of 2-mercaptoethanol and 50 mg of dithiothreitol (Sigma Chemical Co., St. Louis, Mo.) were added, and the suspension was incubated at 37°C for 1 h. This was followed by the addition of 20 mg each of dried preparations of Zymolyase 5000 (5,000 units of activity per g; Sekagaku Kogyo Co., Tokyo, Japan) plus chitinase (450 units of activity per g; Sigma). The suspension was mixed by gentle vortexing, incubated at 37°C, and checked by light microscopy after 2 to 3 h for release of spheroplasts from the hyphal fragments. The sample was then centrifuged (500 × *g*) for 10 min, which partially separated the spheroplasts in the supernatant from the wall and the intact hyphae in the pellet. The spheroplasts were then pelleted by centrifugation at 3,000 × *g* for 10 min, washed three times with 1 M sorbitol–50 mM EDTA (pH 8.0), and resuspended in 1.0 ml of the same solution containing proteinase K (2 mg/ml, 10 to 20 units of activity per mg; Sigma). The sample was immediately mixed with 1% low-melting-point agarose (1:1.5 [vol/vol]) added to wells of sample plug molds (Bio-Rad) and cooled to 4°C. The 1% agarose was prepared in 50 mM sodium citrate buffer which contained 125 mM EDTA and kept at 50°C. The agarose plugs were removed from the molds, placed in NDS buffer (0.5 M EDTA [pH 8.0], 10 mM Tris-HCl [pH 9.5], 1% N-lauroylsarcosine) (30), and incubated at 50°C for 24 h. The plugs were then washed three times with 50 mM EDTA and stored at 4°C until subjected to pulsed-field electrophoresis.

In the agarose spheroplast procedure the mycelial phase of *C. immitis* was grown and isolated as described above. Growth of *N. crassa* mycelia was done in GYE medium at 24°C for 18 h, and spheroplasts were produced in agarose as described by Orbach et al. (26). For *C. immitis*, approximately 40 mg (wet wt) of mycelia was resuspended in 1.0 ml of 125 mM EDTA–50 mM sodium citrate to which 10 mg of chitinase (200 to 600 units of activity per g; Sigma) was added. The suspension was immediately mixed with 1.5 ml of molten 1% low-melting-point agarose (at 55°C in 125 mM EDTA–50 mM sodium citrate), which was added to plug molds and cooled to 4°C for 20 min. Spheroplasts were produced in the agarose by incubation of the plugs in 50 mM sodium citrate (pH 5.7)–0.4 M EDTA (pH 8.0)–1% 2-mercaptoethanol at 37°C for 24 h. The plugs were then placed in fresh solution as described above and incubated under the same conditions for an additional 24 h. To lyse the spheroplasts, plugs were washed three times with 50 mM EDTA (pH 8.0) and then incubated in NDS buffer which contained 2 mg of proteinase K per ml at 50°C for 24 h. The plugs were subsequently rinsed three times with 50 mM EDTA (pH 8.0) and stored at 4°C in preparation for electrophoresis.

Light and electron microscopy. Spheroplasts prepared by the above procedures were examined by light or electron microscopy after incubation of mycelia with the zymolyase-chitinase reaction mixture (the liquid spheroplast procedure) or in agarose plugs after incubation with chitinase (the agarose spheroplast procedure). Samples prepared by the liquid spheroplast procedure were examined by light microscopy with phase contrast and fluorescence illumination. Spheroplast suspensions were fixed for 2 h at room temperature in 2% glutaraldehyde plus 2% paraformaldehyde (Polysciences, Washington, Pa.), prepared in 0.1 M cacodylic acid buffer (pH 7.2). The samples were rinsed two times with buffer and then incubated in a mixture of equal parts of mithramycin and ethidium bromide stains (Sigma) (7) at the following concentrations: 50 μg of mithramycin per ml in 7.5 mM $MgCl_2$–12.5% ethanol and 25 μg of ethidium bromide per ml in 0.1 M Tris buffer–0.6% NaCl (pH 7.4). After reaction for 30 min at 4°C, the stained cells were ready for examination by fluorescence microscopy.

Samples prepared by the agarose spheroplast method were chemically fixed for transmission electron microscopy as described above and then postfixed in 1% osmium tetroxide (Polysciences). Subsequent preparatory steps for electron microscopy were the same as those previously described (20).

Pulsed-field electrophoresis. CHEF gel electrophoresis was performed with a model DR II (Bio-Rad) gel apparatus and power supply. A modification of the method of Vollrath and Davis (36) was employed. Agarose gels (0.6 to 0.8%; Sea Kem Gold Agarose; FMC, Rockland, Maine) were prepared in 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA; Bio-Rad) and poured into molds (14.0 by 12.7 cm and 3.5 mm thick). All gels were electrophoresed in 0.5× TBE buffer which was cooled to 9 or 12°C. DNA samples were cut from agarose plugs described above, placed in the gel wells, and sealed with 0.6% low-melting-point agarose. The optimal voltage, switch interval, and total time used for electrophoresis of the *C. immitis* DNA samples were 40 V, 75 min, and 120 h, respectively. Variations in voltage (50 to 140 V), switch interval (5, 20, or 75 min), and duration of run (20 to 160 h) were conducted with selected isolates in attempts to improve separation of their chromosomal bands. The gels were stained with ethidium bromide (0.5 μg/ml) for 30 min at room temperature, destained in distilled water for 30 min, and photographed with transmitted UV light. The lanes on the negatives were scanned with a scanning densitometer (model 1650; Bio-Rad) as previously described (25).

Southern hybridization. Chromosomal DNA fragments were transferred to Zeta-Probe blotting membrane (Bio-Rad) and hybridized to selected DNA probes of *C. immitis* according to the manufacturer's protocol. Briefly, the stained gels were first irradiated for 18 min with UV light (300 nm; model 310 transilluminator; Fotodyne, New Berlin, Wis.) to cleave the chromosomal DNA. Transfer to the membrane was accomplished in 0.4 N NaOH by blotting over a period of 24 h. The membrane was then washed with 2× SSC (0.30 M NaCl plus 0.03 M sodium citrate, pH 7.0), dried by blotting on 3 MM filter paper (Whatman Ltd., Maidstone, England), baked under vacuum in an oven at 80°C for 30 min, and stored. The membranes were first

treated with prehybridization solution (1 mM EDTA plus 7.0% sodium dodecyl sulfate [SDS] in 0.5 M NaH_2PO_4 [pH 7.2]; 150 μl/cm^2 of Zeta-Probe membrane) in sealed plastic bags for 5 min at 65°C. This solution was replaced with a fresh solution of the same composition plus the denatured and labeled DNA probe (see below), and hybridization was performed at 65°C for 18 h. The probes were denatured by boiling (5 min) and labeled by the random hexamer primer method (16) with [α-^{32}P]dATP (>3,000 Ci/mmol; ICN, Costa Mesa, Calif.). Probes with specific activities of >5 × 10^8 cpm/μg were used at a concentration of 10^6 cpm/ml. After hybridization, the membrane was removed from the bag and washed twice with 40 mM sodium phosphate buffer plus 1 mM EDTA–5% SDS and then twice with 1% SDS in the same buffer. Washes were each performed at 65°C for 30 min. The membranes were used to expose autoradiographic film. The same membrane was employed to sequentially test several DNA probes. The hybridized membrane was washed twice with 0.1× SSC plus 0.5% SDS at 95°C for 20 min. This procedure totally removed the labeled probe, and the membrane could then be reacted with another DNA probe.

DNA probes. A ribosomal DNA probe was provided by K.-J. Kwon-Chung (National Institutes of Health, Bethesda, Md.). The pBIR6 plasmid which contained a *Cryptococcus neoformans* rDNA insert (28) was digested with *Hin*dIII to yield an 8.6-kb fragment. The probe was identified in this study as CnR. Three DNA probes from *C. immitis* were used and previously described as the 1.2-kb cDNA which encodes a 34-kDa chymotrypsinlike serine proteinase (12), the 0.2-kb cDNA which encodes a 7.3-kDa T-cell-reactive peptide (20), and a 0.8-kb cDNA isolated from a *C. immitis* cDNA expression library which was screened with rabbit antiserum raised against a purified 120-kDa alkaline β-glucosidase (21). These three *C. immitis* cDNA probes were identified in this study as CiSP, CiTRP, and CiAG, respectively.

Cytofluorometric analysis. Microspectrophotometry was used to estimate total nuclear DNA content of *C. immitis* by measurement of the fluorescence intensity of stained nuclei (40). *C. immitis* arthroconidia were used to inoculate Converse liquid media as previously described (10). The cultures were incubated at 39°C with addition of 20% CO_2 as described previously (10). After 8 h, hydroxyurea (Hu; Sigma) was added to the culture media at a final concentration of 0.2 M. This step was performed to inhibit DNA synthesis, but not cell growth, and thereby to increase the percentage of cells in the G_1 phase of the cell cycle (7). A concentrated Hu stock solution was prepared in Converse medium and filter sterilized. Incubation of the cultures was continued as described above for another 6 h after the addition of Hu. The cells were harvested by centrifugation (7,000 × *g*), and the pellet, which contained approximately 10^8 cells, was resuspended in 70% ethanol at 4°C. After ethanol fixation (1 h), the cells were washed with distilled water, and the pellet was resuspended in 0.1% (wt/vol) ribonuclease A (Sigma) at 37°C for 30 min. The cells were then washed three times with distilled water (4°C) and stained with ethidium bromide (25 μg/ml in 0.1 M Tris-HCl plus 0.6% NaCl [pH 7.4]) on ice for 30 min. The cells were washed with distilled water (three times) and immediately examined by fluorescence microscopy. *Saccharomyces cerevisiae* haploid strain (ATCC 24851) and diploid strain (ATCC 24860) were used as controls for the cytofluorometric analyses. The yeast strains were grown in YEPD medium (Difco Laboratories, Detroit, Mich.) at 25°C for 48 h and then transferred to fresh medium which contained 0.2 M Hu for an additional 6 h. The cells were fixed and stained as described above.

The intensity of fluorescence of the nuclear stain is considered to be approximately proportional to the DNA content (7, 40). Incubation of *C. immitis* with RNase minimized background staining due to intercalation of ethidium bromide with double-stranded RNA (29). Samples were examined with a Zeiss Universal photomicroscope equipped with a 100-W mercury lamp and 395-nm filter (Zeiss III RS) for epifluorescence illumination. Intensity of fluorescence was determined with a model MTI 65 Newicon camera (DAGE-MTI Inc., Albuquerque, N.Mex.) interfaced with an IBAS digital image processing system (Kontron Co., Munich, Germany).

RESULTS

Spheroplast production from *C. immitis* mycelia. The result of treatment of mycelial suspensions with osmotically buffered EDTA and reducing agents followed by incubation with zymolyase plus chitinase (liquid spheroplast procedure) is shown in Fig. 1. Large numbers of spheroplasts together with hyphal fragments were visible in the incubation mixture after 3 h (Fig. 1A). Much of the hyphal wall material was subsequently separated from the spheroplasts by centrifugation. When stained with the mithramycin-ethidium bromide mixture, the isolated cells were shown to be multinucleate (Fig. 1B). A spheroplast isolated in agarose by the second DNA preparative procedure described above is shown in Fig. 1C. The plasma membrane, nucleus, and other organelles are visible. Chitinase together with EDTA and 2-mercaptoethanol was effective in this procedure for digestion of the walls of *C. immitis* mycelia embedded in agarose plugs. This significantly increased the ease of spheroplast production and DNA preparation compared with that by the liquid spheroplast procedure.

Several factors contributed to our success in the preparation of chromosomal DNA in this study. Young mycelial cultures of *C. immitis* 2 to 3 days after inoculation with arthroconidia provided the best DNA yield. Efficient spheroplast production and ease in handling DNA preparations were achieved by the modified agarose-spheroplast method (26). Chromosomal DNA prepared for pulsed-field electrophoresis by this method could be stored in 50 mM EDTA at 4°C for up to 3 months without apparent degradation of the DNA.

Separation of chromosomal DNA by CHEF. We attempted to optimize chromosomal DNA separation in *C. immitis* isolates by variation of specific conditions for CHEF electrophoresis, which included voltage, switch interval, and total duration of the run. The best resolution of chromosomal bands for the majority of isolates examined was achieved with 40 V, switch interval of 75 min, and total duration for electrophoresis of 120 h. Under these running conditions, the electrophoretic karyotypes and staining intensity of the chromosomal bands were reproducible from one preparation to another. The results of CHEF gel electrophoresis and respective densitometric tracings for representative isolates of the pathogen are shown in Fig. 2. The Silveira and UT19 isolates clearly revealed four chromosomal bands (B1 to B4), while the ethidium bromide-stained gels of C634 and L isolates (Fig. 2A) each appeared to have three bands. On the other hand, the densitometric tracing of isolate C634 (Fig. 2B) suggested that a fourth band (B2) was present. By simply decreasing the amount of DNA of C634 applied to the wells, but maintaining the same running conditions, we were able to resolve two upper chromosomal bands for this isolate (Fig. 2C and D). Three other isolates

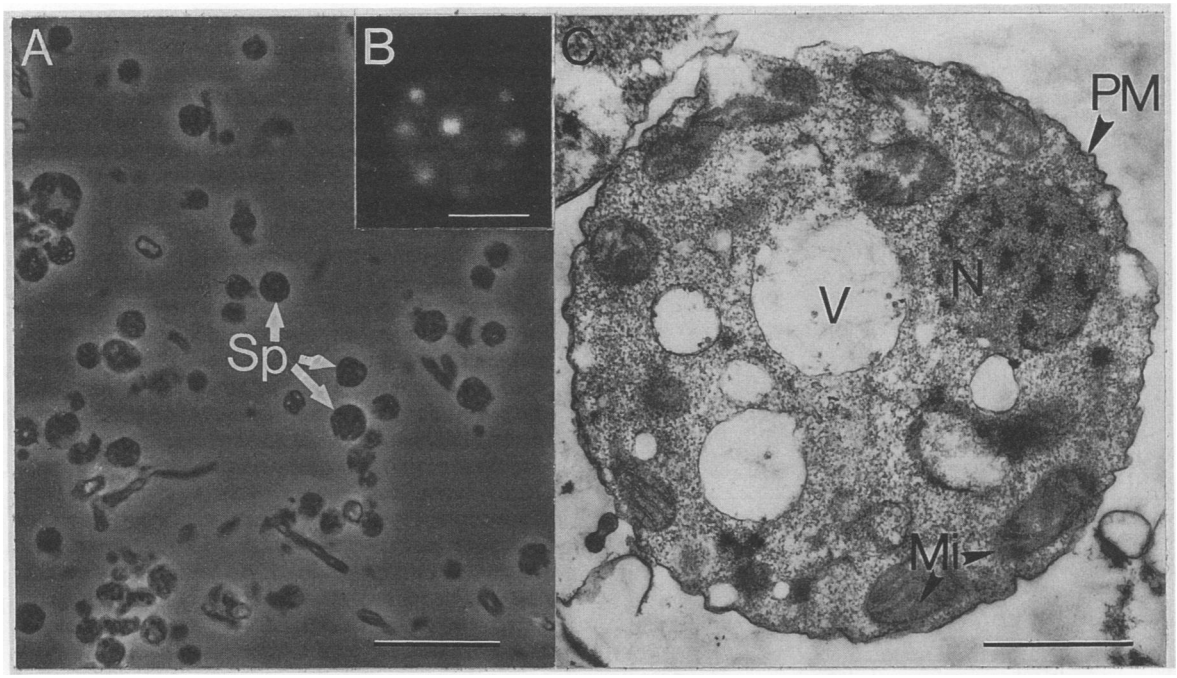

FIG. 1. Spheroplasts produced in suspension by the modified liquid spheroplast procedure (26) (A and B), and by the modified agarose procedure (C). The spheroplast in panel C was examined by electron microscopy. The multinucleate spheroplast in panel B was stained with mithramycin and ethidium bromide. Mi, mitochondria; N, nucleus; PM, plasma membrane; Sp, spheroplasts; V, vacuole. Bars in panels A to C represent 10, 4, and 1 μm, respectively.

(UT17, UT18, and C24) showed closely juxtaposed, high-molecular-weight chromosomal bands (Fig. 3A and B). We were unable to clearly resolve four chromosomal bands in the C735 and L isolates by using the electrophoresis conditions which were optimal for the other samples examined (Fig. 3A and B). An increase in total duration of the pulsed-field electrophoresis to 160 h and maintenance of the same voltage and switch interval resulted in partial separation of two high-molecular-weight chromosomal bands in the C634, C735, and L isolates (Fig. 3C and D). Hybridization experiments with radiolabeled cDNA probes were conducted to further examine the number of chromosomal bands in selected *C. immitis* isolates.

Hybridization of gene probes with different chromosomal bands. Both the ribosomal DNA probe (CnR) and 1.2-kb cDNA probe derived from *C. immitis* (CiSP) hybridized with the lowest-molecular-weight chromosomal band of isolates UT19, C634, and L (Fig. 4). The 0.2-kb cDNA probe (CiTRP) and 0.8-kb cDNA probe (CiAG), on the other hand, distinguished between the two high-molecular-weight chromosomal bands. CiTRP hybridized with the upper chromosomal band of the UT19 isolate and with the upper region of the highest-molecular-weight band of the C634 and L isolates (Fig. 4). CiAG hybridized with the adjacent lower band of UT19 and with the lower region of the high-molecular-weight band in C634. Southern hybridizations with these probes were conducted with chromosomal DNA separations of eight other isolates. The results (not shown) revealed the same assignment of gene probes to respective chromosomal bands as demonstrated by the representative UT19 and C634 isolates in Fig. 4. The CiTRP and CiAG probes appeared to hybridize with approximately the same region of the highest-molecular-weight band of the L isolate (Fig. 4). These same two probes hybridized with the single highest-molecular-weight band of the four chromosome containing Silveira isolate (data not shown).

The fluorescence intensity of chromosome 1 (uppermost gel band) of isolates UT19 and L in Fig. 2 to 4 appeared equal to that of chromosome 4 (lowermost band). Densitometric analysis of the gel separation of the L isolate in Fig. 3D indicated that the uppermost band was actually two chromosomes. To determine whether the lowermost band of the UT19 and L isolates represents one or two chromosomes, CHEF gel separations were conducted under widely varying electrophoretic conditions (Fig. 5A to C). Under each running condition, the migration distance of the lowermost band of the two isolates was approximately the same. No separation of this band into lower-molecular-size components was visible in the results shown Fig. 5A and B. Southern hybridization was conducted between either the rDNA probe (CnR; Fig. 5C and D) or *C. immitis* serine proteinase cDNA (Fig. 5C and E) and CHEF gels run under conditions which optimized separation of low-molecular-size chromosomal bands of these two isolates (Fig. 5C). Only the lowermost band of each isolate was reactive, as demonstrated in Fig. 4 with these same probes. The rDNA probe also hybridized with the *S. pombe* and *S. cerevisiae* standards (Fig. 5D).

Estimate of molecular sizes of chromosomal bands. Chromosomal DNA preparations from *N. crassa* and *S. pombe* were used as standards and subjected to CHEF gel electrophoretic separation under the same conditions as employed for *C. immitis* in Fig. 2. The apparent molecular mass of each chromosomal band was plotted as a function of its distance migrated in the gel (Fig. 6) as described by Cox and coworkers (13). Only the three largest chromosomal bands of *N. crassa* were resolved by the pulsed-field conditions used in this study. The sizes of these three bands were plotted according to published estimates for the three largest

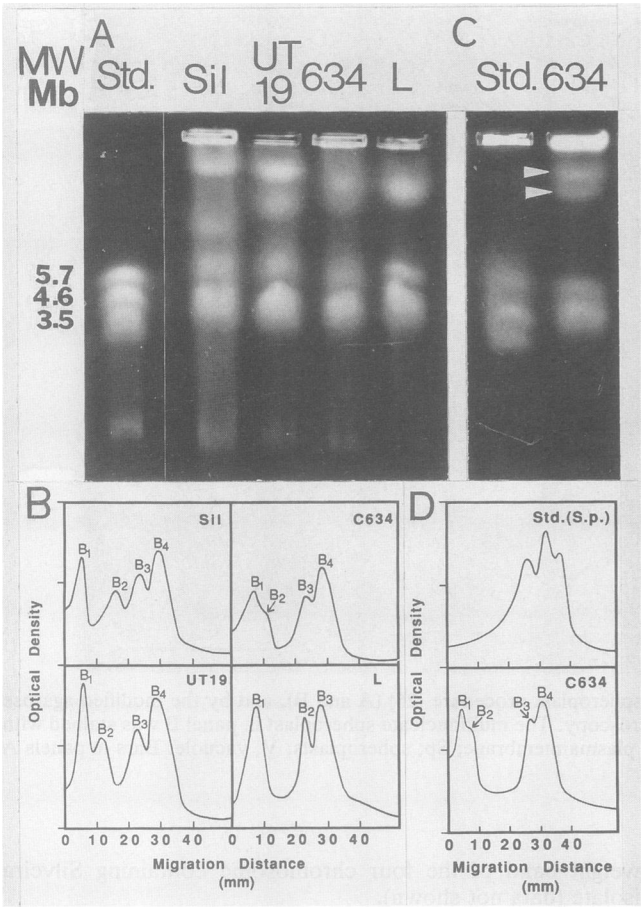

FIG. 2. (A and C) Ethidium bromide-stained CHEF electrophoresis gels of chromosomal DNA preparations of designated strains; (B and D) respective densitometric tracings of chromosomal bands (B1 to B4). Note that only three chromosomal bands (B1 to B3) are resolved in the L isolate. The electrophoresis running conditions were 40 V, 75-min switch interval, and 120-h duration at 9°C. Reduction (approximately 50%) of the C634 DNA sample added to the loading well in panel C permitted resolution of four distinct chromosomal bands (D). The two high-molecular-weight, chromosomal bands of this isolate are identified by arrowheads in panel C. Molecular weights (in megabases) for the *S. pombe* standards (Std.) are indicated to the left of panel A.

chromosomes of this fungus (26). Four smaller chromosomal bands of *N. crassa*, reported to be in the size range of 4 to 6 Mb (26), were not clearly resolved under our electrophoresis running conditions and were not included in the standard curve (Fig. 6). The size range of the *C. immitis* chromosomes (I to IV; Fig. 6A) resolved in this study was approximately 11.5 to 3.2 Mb (Fig. 6B). Most values for migration distance of the *C. immitis* chromosomal bands fell within the near linear portion of the standard curve. Chromosomes III and IV of the *C. immitis* isolates examined showed similar molecular sizes (average of approximately 5 and 3.5 Mb, respectively), except in the case of isolate C24 (approximately 3.5 and 3.2 Mb, respectively; Fig. 3A and B). Greater variability in molecular size of the upper two chromosomal bands (I and II) was demonstrated. For some isolates (e.g., C634, C735, L), the mobilities of these two chromosomes in the pulsed-field electrophoresis gel were nearly identical, which suggested that their molecular sizes were very similar.

Cytofluorometric and electrophoretic estimates of genome size. The cultivation, hydroxyurea exposure, and nuclear stain procedures used in preparation of cells for cytofluorometric analyses permitted adequate and consistent resolution of nuclear DNA (Fig. 7A–D). The arthroconidia of *C. immitis* examined for fluorescence intensity of the ethidium bromide-stained nuclei were slightly swollen after incubation in the Converse medium for 14 h (Fig. 7C and D). The fluorescence intensity of *C. immitis* nuclei (from >100 cells) was compared with that of *S. cerevisiae* haploid and diploid yeast nuclei (Fig. 7E). The latter two strains showed a predicted, approximately 1:2 ratio of relative nuclear DNA content, which was also reported by earlier investigators (24) after ethidium bromide staining. The reported genome size of the haploid strain of *S. cerevisiae* is approximately 13.2 Mb (4). This estimate was used as the basis for calculation of the genome size of *C. immitis*. Comparison of the fluorescence intensity of nuclei in the *S. cerevisiae* strains with the fluorescence intensity of *C. immitis* nuclei in Fig. 7E yielded an estimated genome size for *C. immitis* of 28.2 ± 2.6 Mb. The two isolates of *C. immitis* used in the fluorometric analyses were UT19 and L. The calculated genome size of *C. immitis* based on the addition of the average molecular sizes of chromosomal DNA bands in CHEF gels for nine isolates examined was 29.0 ± 3.0 Mb.

DISCUSSION

Chromosome-size DNA molecules of *C. immitis* were successfully prepared from mycelium-derived, multinucleate spheroplasts. The latter were easily obtained from all isolates examined by a modification of the agarose spheroplast method of Orbach et al. (26). Chromosomes were separated by pulsed-field gel electrophoresis with a CHEF apparatus. Electrophoretic separations were conducted with 12 representative isolates by using different combinations of voltage, switch interval, and duration of run to establish the optimal conditions for resolution of chromosomal bands. We were able to resolve four distinct chromosomes in CHEF gel separations of all but the C634, C735, and L isolates when pulsed-field electrophoresis was conducted at 40 V, with a switch interval of 75 min for a total of 120 h. As indicated, the electrophoretic karyotypes under these conditions were highly reproducible. Differences in fluorescence intensity of the chromosomal bands suggested that not all bands were present in equimolar amounts. It has been suggested that differences in ethidium bromide binding between bands may occur, especially if the total nuclear DNA contains a high amount of A+T (13). The A+T content of *C. immitis* DNA is unknown. Four chromosomes were resolved in C634 by simply adjusting the amount of DNA applied to the gel. However, we were unable to establish the optimal conditions for separation of chromosomal bands in the C735 and L isolates. A similar problem of strain variability and apparent inability to standardize the protocol for CHEF electrophoresis separation of chromosomes has been reported for *C. neoformans* (27). The investigators suggested that other separation methods such as field inversion (3) and orthogonal field alteration gel electrophoresis (OFAGE) (3, 30) may improve the resolution of chromosomal bands. These techniques should also be tested in future studies of electrophoretic karyotypes of *C. immitis*.

Hybridization of selected gene probes with CHEF gel-separated chromosomal bands of the UT19 and C634 isolates provided additional evidence for the presence of four chromosomes in *C. immitis*. The 0.2-kb cDNA probe (CiTRP)

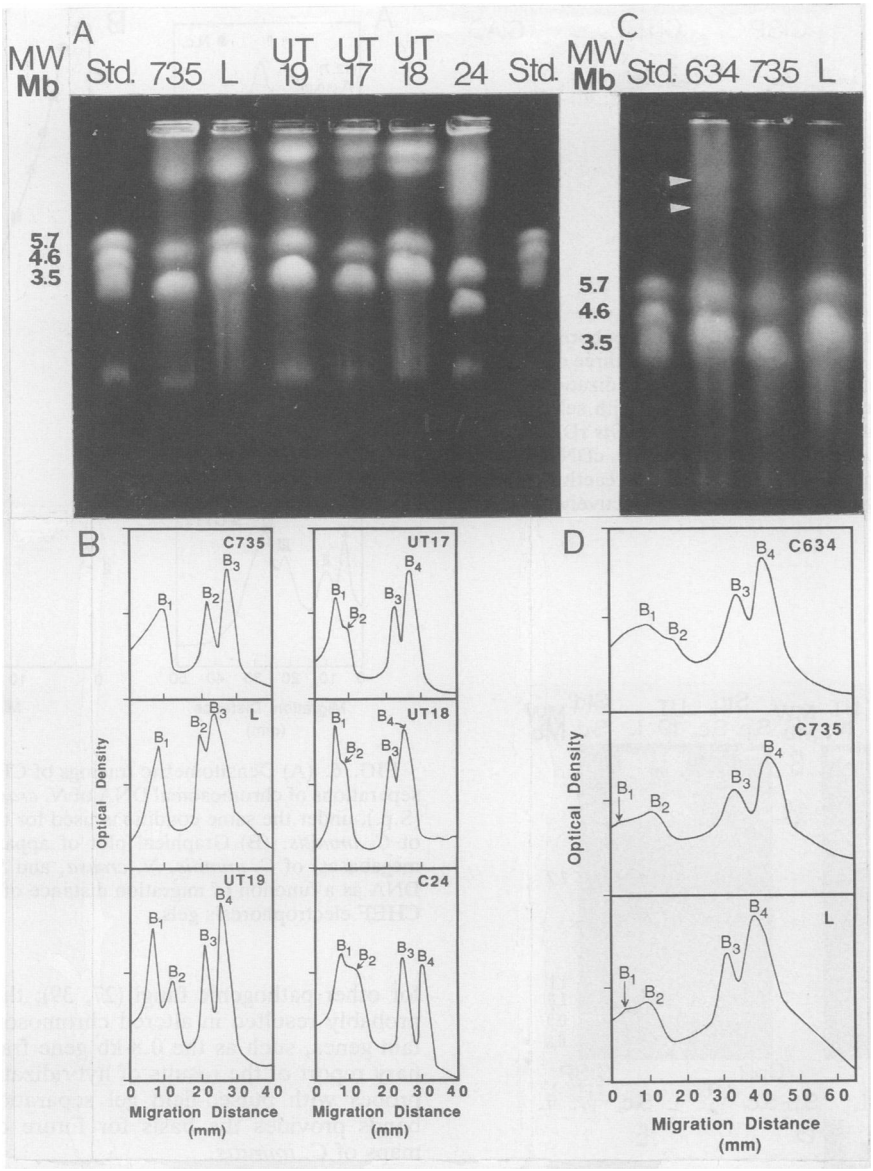

FIG. 3. (A and C) Ethidium bromide-stained CHEF electrophoresis gels of designated strains; (B and D) respective densitometric tracings of chromosomal bands. The electrophoresis running conditions for panels A and B were the same as those described in the legend to Fig. 2. The conditions for panels C and D were 40 V, 75-min switch interval, and 160-h duration at 9°C. Note faintly visible, high-molecular-weight chromosomal bands of C634 (arrowheads) in panel C and suggestion of four chromosomal bands in C735 and L isolates on the basis of densitometric tracings (D) of respective gel lanes (C). Molecular weight standards (in megabases) are indicated to the left of panels A and C.

and 0.8-kb cDNA probe (CiAG) distinguished between two chromosomal bands of high molecular size in UT19 and C634. Similar results were obtained after hybridization of the same DNA probes with almost all other isolates of this pathogen (data not shown). However, in the case of the L isolate, the two probes appeared to react with the same high-molecular-size chromosomal band. The densitometric tracing of the CHEF gel separation of the L isolate, however, indicated the presence of two high-molecular-weight chromosomes of nearly identical gel mobility. The results of our cytofluorometric analysis of ethidium bromide-stained nuclei of the UT19 and L isolates indicated that they have the same genome size. These data support our conclusion that the two isolates have the same number of chromosomes.

The rDNA genes have conserved sequences which account for cross-hybridization between the *C. neoformans* probe (CnR) and *Coccidioides* DNA (27). However, the conserved rDNA genes of *C. immitis* are located on the smallest chromosome in contrast to their localization on the larger chromosomes of *C. neoformans*, *Saccharomyces* species and *Candida* species (1, 27). No gene of *C. immitis* which hybridizes with the approximately 5.7-Mb chromosomal band of the pathogen has so far been isolated. Both the 0.2-kb and 0.8-kb cDNA probes hybridized with the highest-molecular-weight chromosomal band of the Silveira isolate. As stated above, in all other isolates characterized by four distinct bands, these same two probes hybridized with the upper and lower member of the two largest chromosomes, respectively. These observations suggest that DNA rearrangement has occurred in *C. immitis*, as reported

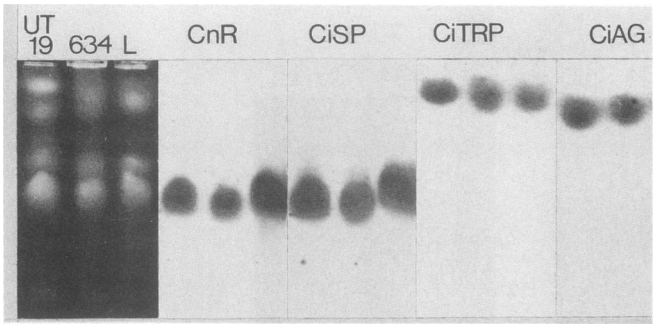

FIG. 4. Ethidium bromide-stained CHEF electrophoresis gel (40 V, 75-min switch interval, 120-h duration at 9°C) of three designated isolates and autoradiographs of four separate hybridizations of the same membrane-transferred chromosomal DNA with selected, radiolabeled probes. The latter include a *C. neoformans* rDNA probe (CnR) and three previously described *C. immitis* cDNA probes which encode a serine proteinase (CiSP), T-cell-reactive peptide (CiTRP), and alkaline β-glucosidase (CiAG), respectively.

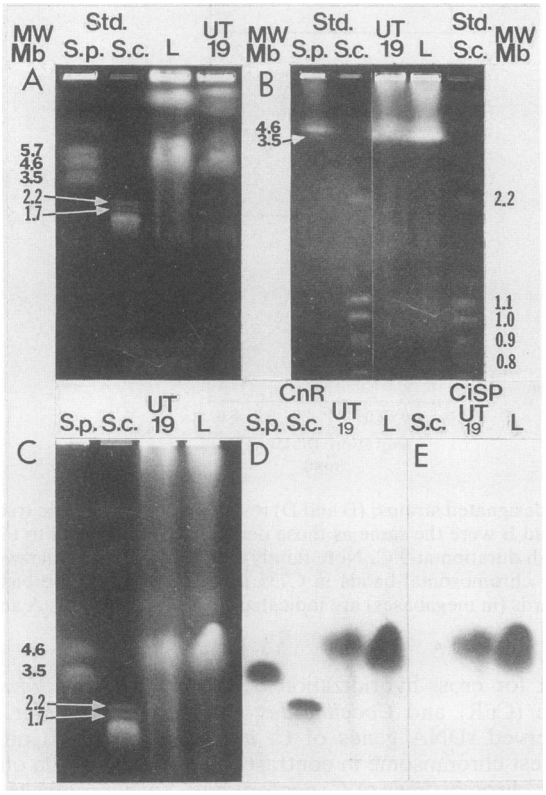

FIG. 5. (A to C) Ethidium bromide-stained CHEF electrophoresis gels of two isolates of *C. immitis* (UT19 and L) run under the following conditions: 0.7% agarose, 60 V, 75-min switch interval, 120-h duration at 9°C (A); 0.8% agarose, 140 V, 5-min switch interval, 20-h duration at 9°C followed by 80 V, 20-min switch interval, and 32-h duration at 9°C (B); and 0.6% agarose, 50 V, 75-min switch interval, 100-h duration at 12°C followed by 35 V, 75-min switch interval, and 35-h duration at 9°C (C). (D and E) Autoradiographs of the gel shown in panel C with rDNA probe (CnR) and *C. immitis* serine proteinase cDNA probe (CiSP), respectively. Molecular weight standards (in megabases) are indicated.

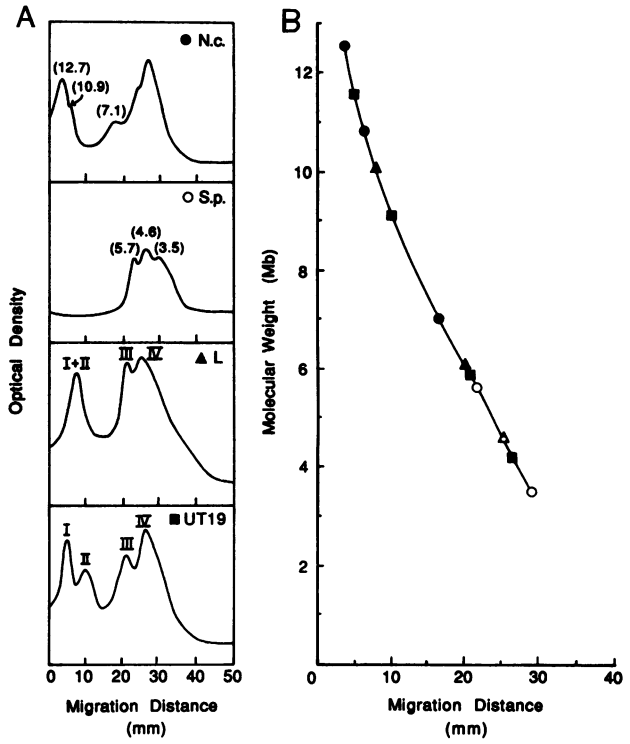

FIG. 6. (A) Densitometric tracings of CHEF electrophoresis gel separations of chromosomal DNA of *N. crassa* (N.c.) and *S. pombe* (S.p.), under the same conditions used for the L and UT19 isolates of *C. immitis*. (B) Graphical plot of apparent molecular size (in megabases) of *C. immitis*, *N. crassa*, and *S. pombe* chromosomal DNA as a function of migration distance of chromosomal bands in CHEF electrophoresis gels.

for other pathogenic fungi (27, 39); the rearrangement has probably resulted in altered chromosomal location for certain genes, such as the 0.8-kb gene fragment. This preliminary report of the results of hybridization of selected DNA probes with pulsed-field gel separations of chromosomal bands provides the basis for future construction of gene maps of *C. immitis*.

The highest-molecular-size chromosomes of *C. immitis* are slightly smaller than the two largest chromosomes reported for *N. crassa* (26). The two smallest chromosomes of *C. immitis* were separated in pulsed-field gels within or close to the size range of the *S. pombe* standards (Bio-Rad). The *N. crassa* and *S. pombe* chromosomal DNA preparations were examined by CHEF gel electrophoresis under the same conditions used for separation of *C. immitis* chromosomal DNA. The results of these analyses permitted construction of a standard curve for calculation of the molecular size of individual *C. immitis* chromosomes. The total DNA content derived from addition of the average molecular weights of chromosomes indicated that the genome size of *C. immitis* is approximately 29 Mb. This is considerably smaller than the estimated genome size of *N. crassa* (45 to 47 Mb [18, 26]) but more than twice as large as the haploid genome of *S. cerevisiae* (4). This latter observation was supported by comparison of the results of our cytofluorometric analyses of *C. immitis* and *S. cerevisiae*. The validity of correlation of fluorescence intensity with total nuclear DNA content was demonstrated by the 1:2 ratio of ethidium bromide intensity for the haploid and diploid strains of *S. cerevisiae*, respec-

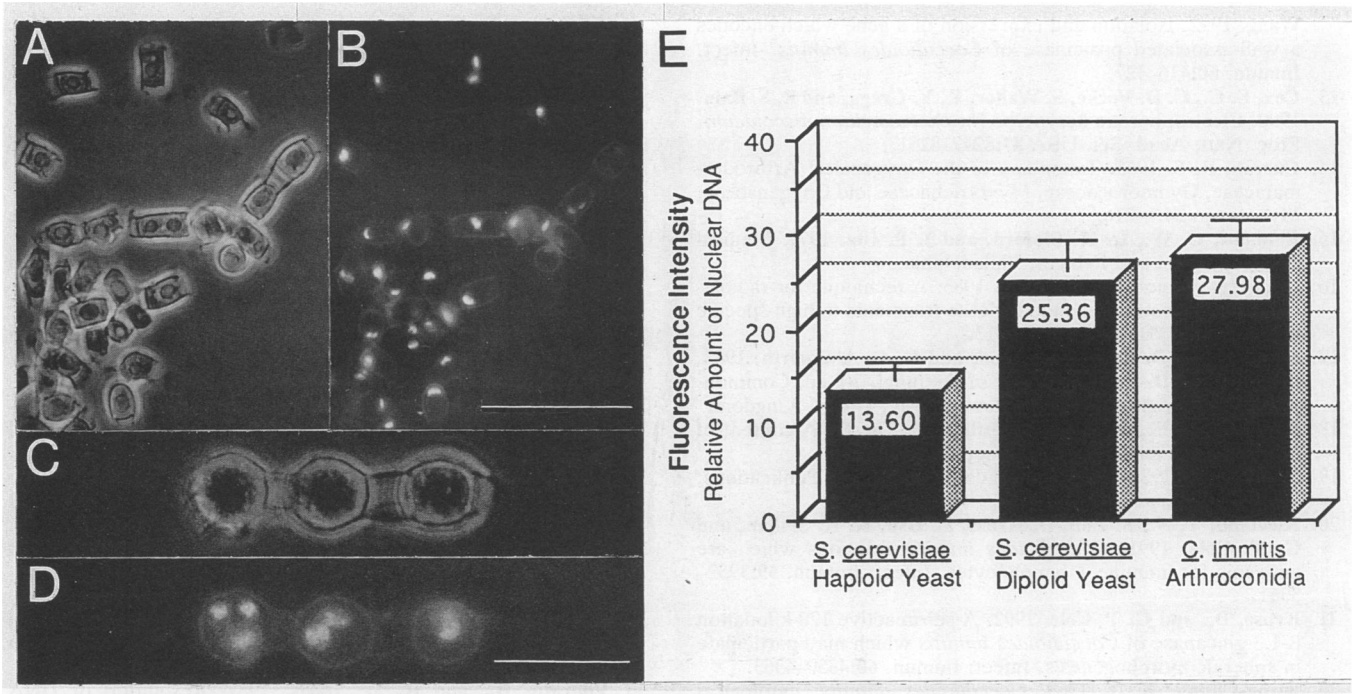

FIG. 7. Arthroconidia isolated from GYE agar cultures at 60 days postinoculation (A and B) and swollen arthroconidia (round cells) isolated from liquid Converse medium plus hydroxyurea 14 h postinoculation (C and D). (B and D) The ethanol-fixed cells were stained with ethidium bromide after incubation with RNase. Bars in panels B and D represent 10 μm. (E) Relative amount of nuclear DNA (based on microspectrophotometric assays of fluorescence intensity of ethidium bromide-stained nuclei) of *S. cerevisiae* haploid and diploid yeast cells and liquid Converse medium-incubated arthroconidia/round cells of *C. immitis*.

tively. These fluorometric data and reported genome size of *S. cerevisiae* were used to estimate the genome size of *C. immitis* by comparative microspectrophotometry. The calculated value (28.2 Mb) was comparable to the estimate derived from pulsed-field gel electrophoresis studies.

The total DNA content of mycelial nuclei based on CHEF gel analyses is the same as that of round cell (young spherule) nuclei based on fluorescence intensity examinations. This suggests that the ploidy of the saprobic and parasitic cycles is the same and the two phases are morphogenetic variations of the asexual cycle of this unusual pathogen. Sun and Huppert (34) reported the presence of four homologous pairs of aceto-orcein-stained chromosomes in round cells formed from arthroconidia after incubation in Converse medium for 24 h. This would suggest that *C. immitis* is a diploid, dimorphic pathogen. Confirmation of its ploidy awaits linkage experiments and genetic studies of selected mutants (38). The nuclear cycle of *C. immitis*, however, remains an enigma. Mature arthroconidia contain 2 to 5 nuclei, while the typical round cell at approximately 8 h after incubation in Converse medium contains a single, large nucleus. The latter is approximately twice the diameter of the nuclei in mature arthroconidia and coenocytic spherules (8, 34). Attempts are under way to synchronize production of the uninucleate round cells and examine the significance of this stage in the nuclear cycle of *C. immitis*.

ACKNOWLEDGMENTS

We are grateful to R. M. Brown for assistance with the cytofluorometric analyses and to D. Brown of the Cell Research Institute for provision of the electron-microscopic facilities.

This study was supported by Public Health Service grant AI19149 from the National Institute of Allergy and Infectious Diseases.

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