Molecular and Cellular Biology, Dec. 1999, p. 7995–8002
Vol. 19, No. 12
Copyright © 1999, American Society for Microbiology. All Rights Reserved.

Mycoplasmal Infections Prevent Apoptosis and Induce Malignant Transformation of Interleukin-3-Dependent 32D Hematopoietic Cells

SHAW-HUEY FENG, SHIEN TSAI, JOSE RODRIGUEZ, AND SHYH-CHING LO*
American Registry of Pathology, Department of Infectious and Parasitic Disease Pathology, Armed Forces Institute of Pathology, Washington, D.C. 20306

Received 15 July 1999/Returned for modification 18 August 1999/Accepted 30 August 1999

32D cells, a murine myeloid cell line, rapidly undergo apoptosis upon withdrawal of interleukin-3 (IL-3) supplement in culture. We found that 32D cells, if infected by several species of human mycoplasmas that rapidly activated NF-κB, would live and continue to grow in IL-3-depleted culture. Mycoplasma-infected cells showed no evidence of autocrine production of IL-3. Pyrroldine dithiocarbamate (PDTC) blocked activation of NF-κB and led to prominent cell death. Heat-killed mycoplasmas or mycoplasmal membrane preparations alone could support continued growth of 32D cells in culture without IL-3 supplement for a substantial period of time. However, upon removal of heat-inactivated mycoplasmas, 32D cells quickly became apoptotic. In comparison, live Mycoplasma fermentans or M. penetrans infection for 4 to 5 weeks induced malignant transformation of 32D cells. Transformed 32D cells grew autonomously and no longer required support of growth-stimulating factors including IL-3 and mycoplasmas. The transformed 32D cells quickly formed tumors when injected into nude mice. Karyotyping showed that development of chromosomal changes and trisomy 19 was often associated with malignant transformation and tumorigenicity of 32D cells. Mycoplasmal infections apparently affected the fidelity of genomic transmission in cell division as well as checkpoints coordinating the progression of cell cycle events.

Mycoplasmas are a heterogeneous group of the smallest organisms capable of self-replication. Mycoplasmas can cause a wide variety of diseases in animals. Some mycoplasmas cause respiratory or urogenital diseases in humans, but others chronically colonize our respiratory and urogenital tracts without apparent clinical significance. In this respect, wall-free mycoplasmas are among the few prokaryotes that can grow in close interaction with mammalian cells, often silently for a long period of time. However, prolonged interactions with mycoplasmas with seemingly low virulence could, through a gradual and progressive course, significantly affect many biological properties of mammalian cells.

Using a murine embryonic (C3H) cell system, we demonstrated that chronic infection by mycoplasmas induced chromosomal instability as well as malignant transformation of mammalian cells. This mycoplasma-mediated onco-genic process had a long latency and demonstrated distinct multistage progression. Overexpression of H-ras and c-myc oncogenes was found to be closely associated with both the initial reversible and the subsequent irreversible states of the mycoplasma-mediated transformation in C3H cells. We have developed a new paradigm for neoplastic processes based on our in vitro studies. We hypothesize that chronic infection or colonization by certain mycoplasmas may gradually induce malignant transformation and promote tumorous growth of mammalian cells.

It is important to note that previous studies reported isolation of mycoplasmas from human leukemic bone marrow (1, 7, 10, 14, 21). A majority of the mycoplasma population isolated was identified as Mycoplasma fermentans. Furthermore, experimental inoculation of M. fermentans induced leukemia in nude mice (20). However, the mycoplasma onco-gensis hypothesis failed to advance because although mycoplasma was isolated most frequently from patients with leukemia, the same mycoplasma could also be found in nonleukemic children or adults (19). Decades later, our understanding of chronic infections, cancer latency, and cancer-associated microbes has changed significantly. The previously described evidence of latent mycoplasmal infection in bone marrow and our findings that chronic infections by mycoplasmas could be associated with a unique form of pathogenesis including cell transformation prompted us to reexamine the mycoplasmal effects on malignant transformation of hematopoietic cells. Growth of murine myeloid 32D cells depends on the continuous induction of interleukin-3 (IL-3) (12, 13). Differing from other IL-3-dependent cells such as FD cells, 32D cells remain under strict regulation by the growth signaling of IL-3 and rarely undergo spontaneous transformation or become IL-3 independent. Withdrawal of IL-3 supplement in culture rapidly induces the 32D cells to undergo apoptosis, and more than 80% of the cells die within 4 to 5 days. The growth of 32D cells is regulated closely by growth factor signaling, which provides an ideal model system to study the transforming effects of mycoplasmas.

Remarkably, we found that infections by several species of human mycoplasmas, but not all species tested, would effectively prevent 32D cells from undergoing apoptosis in culture without IL-3 supplement. The mycoplasma-infected 32D cells continued to grow without the induction of IL-3 growth signaling. Moreover, after a period of 4 to 5 weeks of infection by M. fermentans or M. penetrans, 32D cells gradually underwent malignant transformation and no longer required the continued presence of mycoplasmas for growth in the IL-3-free culture. These 32D cells grew autonomously and became highly transformed.
tumorigenic when injected into nude mice. This in vitro model system allowed us to explore mycoplasma-mediated molecular mechanisms that rescue cells from apoptosis and induce continuous cell growth. We also studied the machinery that could lead to malignant transformation in 32D cells chronically infected by mycoplasmas.

MATERIALS AND METHODS

Mycoplasmas and cell culture. M. fermentans PG18 (ATCC 19989), M. penetrans PG18 (ATCC 23064), M. genitalium ATCC 33530, M. pneumoniae ATCC 15531, M. orale ATCC 23714, and M. pirum (kindly provided by J. G. Tully, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were grown aerobically in SP-4 broth medium. The 32D cell line (kindly provided by Jaclyn H. Pierce, National Cancer Institute, National Institutes of Health) is an IL-3-dependent, nontumorigenic cell line that has an undifferentiated myeloid phenotype and a normal diploid karyotype. The cell line was maintained in RPMI culture medium containing 15% fetal calf serum and 5% WEHI-3B conditioned medium (also provided by Jaclyn H. Pierce).

Infection of 32D cells with mycoplasmas. 32D cells were transferred to culture medium free of IL-3 then inoculated with various species of Mycoplasma at a ratio of 1,000 color change units/cell. To determine the growth kinetics of 32D cells in cultures infected with mycoplasmas, cell cultures were initiated at 2 × 10^4 cells/ml. Viable cells stained with trypan blue were examined and counted every 2 to 3 days in a hemocytometer. Cell densities of cultures were adjusted to 2 × 10^5 cells/ml when subcultured.

Heat inactivation of mycoplasmas. Mycoplasmas were cultured in SP-4 medium to the growth phase. After a culture sample was taken for titration, mycoplasma cultures were incubated in a water bath at 70°C for 30 min. Heat-treated mycoplasmas were tested by culture to ensure complete inactivation of the organisms and centrifuged in a microcentrifuge at 12,000 rpm for 20 min. Mycoplasma pellets were then resuspended in RPMI 1640 medium at 1/10 of the original volume. These suspensions of heat-killed mycoplasmas were kept frozen at -70°C until needed. The amount of heat-inactivated mycoplasmas added to the cell cultures equaled 1,000 color change units/ml.

Preparation of nuclear proteins. Nuclear proteins were prepared by the method of Schreiber et al. (27). Typically, 5 × 10^6 to 1 × 10^7 32D cells were washed with 10 ml of Tris-buffered saline and pelleted. The pellet was resuspended in 1 ml of Tris-buffered saline and pelleted again by spinning for 15 min in a microcentrifuge. Tris-buffered saline was removed, and the cell pellet was resuspended in 0.8 ml of cold buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 1.5 mM pepstatin). The cells were incubated on ice for 15 min, after which 50 μl of a 10% solution of Nonidet NP-40 was added and the tube vigorously vortexed for 10 s. The homogenate was centrifuged at 30,000 × g in a microcentrifuge, and the supernatant was removed. The nuclear pellet was resuspended in 100 μl of ice-cold buffer C (20 mM HEPES [pH 7.5], 4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 μg/ml phenylmethylsulfonyl fluoride, 10 mM leupeptin, and 1.5 mM pepstatin), and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microcentrifuge at 4°C, and the supernatant was frozen in aliquots at -70°C. Protein determination was performed by using a Bio-Rad DC protein assay kit.

EMSA. Electrophoretic mobility shift assay (EMSA) was performed as described by Vincenti et al. (32). A double-stranded oligonucleotide containing a mouse tumor necrosis factor alpha enhancer located at -510 bp from the start of transcription to the tumor necrosis factor alpha coding region was used for the binding assay. The sequences of the two strands of the oligonucleotide with an ATG binding site were 5'-GCG AAC TAC TGA GTC GGC CTT-3' and 3'-GTT TGT CCC CCG AAA GGG AGG AG-5'. In the assay, a 32P-labeled oligonucleotide fragment (100,000 cpm) was mixed with 2 μg of nuclear protein in a total volume of 20 μl. The reaction mixtures were incubated at room temperature for 30 min in the range of 5 × 10^6 to 4 × 10^7 cells/ml. Viable cells were examined by trypan blue staining and counted in a hemocytometer. Growth curves were plotted based on cumulative cell numbers.

RESULTS

Mycoplasmal infections induce continued growth of 32D cells in IL-3-deprived culture. Growth of 32D cells was strictly dependent on IL-3. Removal of IL-3 from the culture caused immediate growth arrest and apoptosis of 32D cells; about 75% of the cells died by day 4. Surprisingly, infections by mycoplasmas effectively prevent 32D cells from undergoing apoptosis in the IL-3-deprived culture. As a typical example, Fig. 1 shows that infection by *M. fermentans* PG18 rescued 32D cells from apoptosis and induced continued cell growth in the IL-3-free culture. In this study, all mycoplasma-infected 32D cells that continued to proliferate were maintained in culture for isolating mycoplasmas (5). Additionally, DNAs were isolated from the cell cultures and assayed for the presence of mycoplasmal DNA by PCR using primers specific for *M. fermentans* and *M. penetrans* (34) or specific 16S rRNA genes (11).

Tumorigenicity in nude mice. Normal, infected, and transformed 32D cells were harvested from cultures and suspended in a small volume (less than 1 ml) of phosphate-buffered saline (PBS). About 5 × 10^5 cells in 0.2 ml PBS were injected subcutaneously into each nude mouse (6 to 8 weeks old; Harlan-Sprague Dawley). The animals were carefully monitored for 1 year for tumor formation.

Karyotype analysis. Actively growing 32D cell cultures were incubated in the presence of colcemid (0.05 μg/ml) for 1 h to accumulate metaphase cells. Cells were harvested in hypotonic solution (0.075 M KCl) for 10 to 15 min; cell pellets were fixed in methanol-acetic acid mixture (3:1 vol/vol) and then washed three times in the fresh fixative. Chromosome preparations were stained with Giemsa staining solution or by the trypsin-Giemsa banding method (35).

Fig. 1. *M. fermentans* PG18 infection induces continued cell growth of 32D cells in culture deprived of IL-3. 32D cells initiated at 5 × 10^5 cells/ml in culture medium containing IL-3 or 2 × 10^5 cells/ml in culture medium deprived of IL-3 were infected by *M. fermentans* PG18 at a ratio of 1,000 color change units/cell. To avoid medium depletion and ensure logarithmic growth, infected and noninfected 32D cells cultured in IL-3-containing medium were subcultured with a 1:10 dilution on every 4th day. Infected 32D cells cultured in IL-3-deprived medium were subcultured on days 6, 12, 18, and 28 to maintain a cell density in the range of 5 × 10^6 to 4 × 10^7 cells/ml. Viable cells were examined by trypan blue staining and counted in a hemocytometer. Growth curves were plotted based on cumulative cell numbers.
M. fermentans growth of 32D cells in the IL-3-deprived culture. In addition to for the ability to prevent apoptosis and induce continued
ined nine different strains and species of human mycoplasmas
were found to induce continued growth of 32D cells in IL-3-free culture for more than 3 months. We exam-
ined the ability of M. fermentans PG18, M. penetrans, M. genitalium, M. salivarium, and A25 to rescue 32D cells from undergoing apoptosis in IL-3-free culture.

Mycoplasma-induced transformation of 32D cells by NF-κB and AP-1 nuclear binding activities
Mycoplasma-infected 32D cells do not produce IL-3. The possible explanation that infections by mycoplasmas could induce continued growth of 32D cells in culture without any supplement of IL-3 was that mycoplasma-infected 32D cells were themselves producing IL-3. The newly produced IL-3 would provide autocrine signaling and support growth of 32D cells. Thus, we examined expression of the IL-3 gene in myco-

Mycoplasmas induce NF-κB and enhance AP-1 nuclear binding activities in 32D cells. Recently, various membrane preparations from mycoplasmas were found to be potent activators for NF-κB and AP-1 transcription factors in murine macrophages (8, 9, 25, 26). Since NF-κB was known to have marked antiapoptosis functions (2, 31, 33) we examined by EMSA the NF-κB activities in 32D cells before and after mycoplasmal infections. Nuclear extracts of 32D cells grown in IL-3-free culture by rapidly inducing NF-κB binding activity in the nuclear extract of 32D cells (Table 1). In comparison, infection by M. salivarium or M. pirum, which failed to support continued growth of 32D cells in IL-3-free culture, could not elicit the same nuclear factor response (Fig. 2A and Table 1).

There was a basal level of activity for transcription factor AP-1 detected by EMSA in the nuclear extracts of control 32D cells grown in culture supplemented with IL-3. Transferring control 32D cells to IL-3-free culture resulted in a rapid loss of all AP-1 binding activity in the nuclear extracts. As described above, the 32D cells without active AP-1 soon underwent apoptosis and died in a few days. In comparison, 32D cells infected by M. penetrans, M. pneumoniae, M. genitalium, and M. orale could support continued growth of 32D cells in IL-3-free culture by rapidly inducing NF-κB binding activity in the nuclear extract of 32D cells (Table 1). In comparison, infection by M. salivarium or M. pirum, which failed to support continued growth of 32D cells in IL-3-free culture, could not elicit the same nuclear factor response (Fig. 2A and Table 1).

There was a basal level of activity for transcription factor AP-1 detected by EMSA in the nuclear extracts of control 32D cells grown in culture supplemented with IL-3. Transferring control 32D cells to IL-3-free culture resulted in a rapid loss of all AP-1 binding activity in the nuclear extracts. As described above, the 32D cells without active AP-1 soon underwent apoptosis and died in a few days. In comparison, 32D cells in-

TABLE 1. Activation of NF-κB, continued cell growth in IL-3-free cultures, and subsequent malignant transformation of 32D cells following mycoplasmal infections

| Presence of IL-3 in medium | Infection             | NF-κB<sup>a</sup> (within 2 days) | AP-1<sup>a</sup> (within 2 days) | Support of continuous cell growth | Eventual cell transformation | NF-κB in transformed cells | AP-1 in transformed cells | Tumorigenicity of transformed cells |
|---------------------------|----------------------|----------------------------------|---------------------------------|----------------------------------|-----------------------------|---------------------------|---------------------------|---------------------------------|
| +                         | None                 | +                                | +                               | +                                | NA<sup>b</sup>              | NA<sup>b</sup>              | NA<sup>b</sup>              | NA<sup>b</sup>                  |
| +                         | None                 | +                                | +                               | +                                | -                           | -                         | -                         | -                               |
| +                         | M. fermentans PG18   | +                                | +                               | +                                | NA<sup>b</sup>              | NA<sup>b</sup>              | NA<sup>b</sup>              | NA<sup>b</sup>                  |
| +                         | M. fermentans A25    | +                                | +                               | +                                | NA<sup>b</sup>              | NA<sup>b</sup>              | NA<sup>b</sup>              | NA<sup>b</sup>                  |
| +                         | M. fermentans incognitus | +                              | +                               | +                                | -                           | -                         | -                         | -                               |
| +                         | M. penetrans         | +                                | +                               | +                                | NA<sup>b</sup>              | NA<sup>b</sup>              | NA<sup>b</sup>              | NA<sup>b</sup>                  |
| +                         | M. salivarium        | +                                | +                               | +                                | ND<sup>c</sup>              | ND<sup>c</sup>              | ND<sup>c</sup>              | ND<sup>c</sup>                  |
| +                         | M. genitalium        | +                                | +                               | +                                | ND<sup>c</sup>              | ND<sup>c</sup>              | ND<sup>c</sup>              | ND<sup>c</sup>                  |
| +                         | M. pneumoniae        | +                                | +                               | +                                | ND<sup>c</sup>              | ND<sup>c</sup>              | ND<sup>c</sup>              | ND<sup>c</sup>                  |
| +                         | M. orale             | +                                | +                               | +                                | ND<sup>c</sup>              | ND<sup>c</sup>              | ND<sup>c</sup>              | ND<sup>c</sup>                  |
| +                         | M. pirum             | +                                | +                               | -                                | -                           | -                         | -                         | -                               |

<sup>a</sup> Induction of transcriptional factor NF-κB detected in the nuclear extract of 32D cells.
<sup>b</sup> Binding activity of AP-1 detected in the nuclear extract of 32D cells.
<sup>c</sup> NA, not applicable.
<sup>d</sup> ND, not done.

FIG. 2. Mycoplasmas induce NF-κB and enhance AP-1 nuclear binding activities in 32D cells. 32D cells infected with M. fermentans PG18 (PG18), M. salivarium (MS), and A25 for 24 h in cultures with or without IL-3. Nuclear extracts prepared from 32D cells with or without mycoplasma infection were analyzed by EMSA for binding to 32P-labeled oligo-nucleotides with binding sites for NF-κB (A) or AP-1 (B).
fected by mycoplasmas for 24 h in IL-3-free culture produced a very high level of AP-1 binding activity (Fig. 2B). In this study, all mycoplasmas tested appeared to have the ability to markedly induce AP-1 activity in 32D cells, including those that failed to support continued growth of 32D cells in IL-3-free culture conditions. For example, 32D cells infected by *M. salivarium* produced as much of the active form of AP-1 as 32D cells infected by *M. fermentans* (Fig. 2B).

**Interference with induction of NF-κB binding activity in mycoplasma-infected 32D cells results in cell death.** Although infections by mycoplasmas could rapidly activate both NF-κB and AP-1 transcription factors in 32D cells, activation of NF-κB appeared to be more closely correlated with IL-3-independent mycoplasma-induced cell growth. To further explore the role that NF-κB might play in preventing apoptosis and in induction of continued cell growth in the absence of IL-3 signaling, we treated 32D cells with pyrrolidine dithiocarbamate (PDTC; Sigma), a potent and specific inhibitor of NF-κB activation. While pretreatment with 100 nM PDTC had little effect, pretreatment with 1,000 nM completely blocked the activation of NF-κB in 32D cells induced by *M. fermentans* PG18 infection (Fig. 3). PDTC at the concentration of 100 nM had no effect on growth of 32D cells induced by the mycoplasma. On the other hand, consistent with the lack of NF-κB activation, mycoplasma-infected 32D cells treated with 1,000 nM PDTC failed to grow and quickly died. In a separate study, treatment of *M. fermentans* incognitus-infected 32D cells with 500 and 1,000 nM PDTC blocked more than 70 and 85% of NF-κB activation, respectively (Fig. 3). Growth of 32D cells induced by infections of the mycoplasma for 2 days was inhibited about 30% by 500 nM and 70% by 1,000 nM PDTC (Fig. 3). PDTC by itself at the concentration of 1,000 nM completely blocked the activation of NF-κB in 32D cells induced by *M. fermentans* PG18 infection (Fig. 3). PDTC at the concentration of 100 nM had no effect on growth of 32D cells induced by the mycoplasma. On the other hand, consistent with the lack of NF-κB activation, mycoplasma-infected 32D cells treated with 1,000 nM PDTC failed to grow and quickly died. In a separate study, treatment of *M. fermentans* incognitus-infected 32D cells with 500 and 1,000 nM PDTC blocked more than 70 and 85% of NF-κB activation, respectively (Fig. 3). Growth of 32D cells induced by infections of the mycoplasma for 2 days was inhibited about 30% by 500 nM and 70% by 1,000 nM PDTC (Fig. 3). PDTC by itself at the concentration of 1,000 nM did not seem toxic or affect the growth of 32D cells supported by IL-3.

Heat-inactivated mycoplasmas and mycoplasmal lipid-associated membrane proteins (LAMPs) can induce IL-3-independent growth of 32D cells. To examine whether infections by live mycoplasmas were required to induce the IL-3-independent growth of 32D cells, we first heat inactivated the mycoplasmas and then examined their ability to induce continued growth of 32D cells in IL-3-free culture. Figure 4A shows that introduction of heat-inactivated *M. fermentans* incognitus or A25 effectively prevented cell death and induced growth of 32D cells in

**FIG. 3.** Interference with induction of NF-κB binding activity results in cell death in mycoplasma-infected 32D cells. 32D cells were treated with PDTC at various concentrations for 1 h prior to transfer to IL-3-free culture medium and infection by *M. fermentans* PG18 (PG18; lanes 2 to 5) or incognitus (Mi; lanes 8 to 10). As positive controls (lanes 1 and 7), 32D cells without PDTC pretreatment were transferred to IL-3-free culture medium and infected with PG18 or Mi, respectively. As a PDTC control (lane 11), 32D cells were treated with 1,000 nM PDTC for 1 h and then cultured in IL-3-containing medium. As a negative control (lane 6), 32D cells were transferred to IL-3-free culture medium without mycoplasma infection. After 2 days, cell numbers were counted, and nuclear extracts were prepared and examined for NF-κB binding activity. Percent NF-κB binding activity was calculated based on the counts per minute of the protein-bound 32P-labeled oligonucleotide bands quantitated by a Storm 860 scanner. In the PG18-infected group, the activity in lane 1 was used as 100%, and values for lanes 2 to 6 were calculated as percentages of this level. In the Mi-infected group, lane 7 represents 100% activity, and values for lanes 8 to 11 were calculated as percentages of this level. Similarly, percent cell numbers were calculated as percentages of the cell number of PG18-infected, non-PDTC-treated 32D cells (lane 1) in the PG18-infected group (lanes 2 to 6) and to that of Mi-infected, non-PDTC-treated 32D cells (lane 7) in the Mi-infected group (lanes 8 to 11). When the differences between the PDTC-treated cells and the non-PDTC-treated mycoplasma-infected cells were less than 5%, percent binding activity and percent cell number were designated 100%.

**FIG. 4.** Heat-inactivated mycoplasmas (A) or mycoplasmal LAMPs (B) induce continued growth of 32D cells in IL-3-deprived culture. *M. fermentans* A25 and incognitus were heat inactivated by treating the cultures at 70°C for 30 min. Heat-inactivated mycoplasmas, or SP-4 broth medium or PBS used as a control, were added to 32D cells after the cells were transferred to IL-3-deprived cultures. Cell numbers were counted on days 3, 5, and 7. LAMPs prepared from *M. fermentans* or *M. penetrans* were heat inactivated by treating the cultures at 70°C for 30 min. Heat-inactivated mycoplasmas, or SP-4 broth medium or PBS used as a control, were added to 32D cells after the cells were transferred to IL-3-deprived cultures. Cell numbers were counted on days 3, 5, and 7.
growth during this period. The nonviable organisms, however, were serially diluted to low concentration and were no longer detectable in culture after several subsequent fresh medium replenishments. The increase of cell number in the culture appeared to slow down significantly after 3 weeks. Examination of the culture revealed that many 32D cells were apparently dying while many others continued to grow. After 7 weeks, rapid cell growth resumed in the culture free of IL-3. PCR analyses confirmed the absence of M. fermentans in the continuously growing 32D cell culture without IL-3 supplement. In addition to M. fermentans PG18, we observed similar transformation of strictly IL-3-dependent 32D cells into autonomously growing cells after 4 to 5 weeks of infections by the M. fermentans A25 and incognitus as well as M. penetrans (Table 1). All of these mycoplasmal agents were eradicated from the 32D cell cultures by 3 weeks of antibiotic treatment.

**Mycoplasma-transformed 32D cells do not have the active nuclear factors of NF-κB and AP-1.** Our earlier study showed rapid activation of NF-κB by mycoplasmas appeared to be essential in preventing 32D cells from undergoing apoptosis in IL-3-free culture. If the continued presence of live mycoplasmas was no longer required for the continued survival of transformed 32D cells in IL-3-free culture, had 32D cells constitutively activated NF-κB or AP-1 following transformation? When we examined NF-κB and AP-1 binding activities by EMSA in nuclear extracts of these mycoplasma-transformed 32D cells that were growing autonomously, we found no positive binding activity of NF-κB or AP-1 (Table 1). Infections by live mycoplasmas for a few weeks apparently activated a new growth-stimulating pathway, different from those of IL-3 signaling or NF-κB activation, in these autonomously growing cells.

**Mycoplasma-transformed 32D cells have abnormal karyotypes.** In C3H murine embryonic cells, an irreversible form of malignant transformation induced by chronic infection of M. fermentans or M. penetrans was found to be associated with development of chromosomal changes (30). We examined alteration of chromosomes in 32D cells following mycoplasma infections and particularly in 32D cells that were transformed and grew autonomously in culture free of growth-stimulating mycoplasmas and IL-3 supplement. Cytogenetic analysis showed that a great majority of the control IL-3-dependent 32D cells had a total of 34 chromosomes with 7 abnormal chromosomes (Table 2) instead of the normal mouse diploid complement of 40 chromosomes. Five of the seven abnormal chromosomes, M1 [rob(2;17)], M2 [rob(3;10)], M3 [rob(4;12)], M4 [rob(12;16)], and M5 [rob(13;19)], were Robertsonian translocation chromosomes; the other two abnormal chromosomes, M6 and M7, appeared to be derivative chromosomes of complex translocation. The M6 chromosome was derived from translocation between chromosomes 9 and 14 [t(9;14)]; the M7 chromosome was chromosome 10 with a very small piece of additional chromosomal material on its centromere. The additional chromosomal material appeared to be a portion of chromosome 14 that is also involved in the translocation with chromosome 9. The representative karyotype of 32D cells is presented in Fig. 6. Infections by various strains of M. fermentans or M. penetrans for 4 to 5 weeks in culture supplemented with IL-3 caused few chromosomal changes in 32D cells. In contrast, 32D cells infected by these mycoplasmas for 4 to 5 weeks in culture without supplemental IL-3 showed significant chromosomal alteration (Table 2). Nearly 50% (23 of 51) of 32D cells infected by M. fermentans PG18 gained an additional chromosome. Interestingly, half (12 of 23) of the cells that gained an additional chromosome had trisomy 19; the other half had an extra copy of various chromosomes. About 6% (3 of 52) of the 32D cells...
infected by *M. fermentans* incognitus for 4 weeks also gained an additional chromosome 19. Most strikingly, more than 95% (52 of 54) of the 32D cells infected by *M. penetrans* GTU-54 for 3 to 4 weeks in culture free of IL-3 had trisomy 19 (Table 2). As described earlier, ciprofloxacin treatment to eradicate mycoplasmas from the culture of mycoplasma-infected 32D cells selected population(s) of truly transformed cells, i.e., cells that were capable of growing autonomously without support of IL-3 or continued presence of growth-stimulating mycoplasmas. Karyotypic analysis of the truly transformed cells induced by either *M. fermentans* or *M. penetrans* showed that populations of 32D cells with trisomy 19 often prevailed (Table 2). All 32D cells transformed by a period of *M. fermentans* PG18 infection (4 weeks) and maintained in IL-3-free culture following ciprofloxacin treatment (32D/IL-3⁻/PG18⁻ culture) either had 34 chromosomes with 8 abnormal chromosomes or 35 chromosomes with an additional copy of chromosome 19. Those cells with 34 chromosomes, in addition to the original 7 abnormal chromosomes, had a new abnormal rob(19;19) chromosome. Thus, both populations of 32D/IL-3⁻/PG18⁻ cells actually had trisomy 19. All 32D cells transformed by a period of *M. fermentans* incognitus infection (4 weeks) and maintained in IL-3-free culture following treatment with ciprofloxacin (32D/IL-3⁻/Mi⁻ culture) had 34 chromosomes. In addition to the original 7 abnormal chromosomes, all of the transformed cells had a new abnormal rob(19;19) chromosome. Thus, they all were trisomy 19. Since more than 95% of 32D cells infected by *M. penetrans* for 4 to 5 weeks (32D/IL-3⁻/Mpe⁺ culture) already had 35 chromosomes with an extra chromosome 19, treatment with ciprofloxacin to eradicate *M. penetrans* in culture free of IL-3 did not alter the cell karyotype. All of the transformed cells that rapidly prevailed after ciprofloxacin treatment to eradicate mycoplasmas (32D/IL-3⁻/Mpe⁻ culture) had trisomy 19 (Table 2).

**Mycoplasma-transformed 32D cells are highly tumorigenic.**

We examined whether the transformed 32D cells induced by mycoplasma infections had become tumorigenic when injected into animals. In this study, $2 \times 10^6$ mycoplasma-infected cells, mycoplasma-free transformed cells, or noninfected control cells were inoculated subcutaneously into each of three nude mice. Similar to the IL-3-dependent 32D control cells, cells infected by various strains of *M. fermentans* for 4 to 5 weeks in culture with or without IL-3 supplement did not form tumors when injected into the animals. As described above, treatment with ciprofloxacin to eradicate *M. fermentans* from the IL-3-

**TABLE 2. Chromosome analysis on 32D cells infected and transformed by *M. fermentans* and *M. penetrans***

| Culture            | No. of cells with chromosome no.: | No. of abnormal chromosomes |
|--------------------|-----------------------------------|-----------------------------|
|                    | 32      | 33      | 34      | 35      | 36      |          |
| 32D cells          | 56      | 2       | 7       |          |          |          |
| 32D/IL-3⁻/PG18⁺    | 2       | 45      | 5       | 7       |          |          |
| 32D/IL-3⁻/PG18⁻    | 1       | 6       | 21      | 23      | 7       |          |
| 32D/IL-3⁻/Mi⁻      | 50      | 11      | 7       |          |          |          |
| 32D/IL-3⁻/Mi⁺      | 2       | 50      | 7       |          |          |          |
| 32D/IL-3⁻/Mpe⁺     | 1       | 17      | 31      | 3       | 7       |          |
| 32D/IL-3⁻/Mpe⁻     | 52      | 2       | 8       |          |          |          |
| 32D/IL-3⁻/Mi⁻      | 2       | 48      | 7       |          |          |          |
| 32D/IL-3⁻/Mi⁺      | 1       | 52      | 1       | 7       |          |          |
| 32D/IL-3⁻/Mpe⁻     | 2       | 50      | 7       |          |          |          |

**DISCUSSION**

IL-3-dependent 32D cells transfected with various onco-genes have served as a model system to study oncogenesis (15). The inherent drawback of this model system is that the cells are transformed artificially by introducing potent transforming genes apart from what naturally transpires. In contrast, our model system using 32D cells examines the transforming ef-

**FIG. 6.** Karyotype of control 32D cells. The majority of 32D cells had 34 chromosomes rather than the normal mouse diploid complement of 40 chromosomes. Among the 34 chromosomes, there were 7 abnormal chromosomes, including 5 Robertsonian translocation chromosomes (M1 [rob(2;17)], M2 [rob(3;10)], M3 [rob(4;12)], M4 [rob(12;16)], and M5 [rob(13;19)]) and 2 derivative chromosomes of translocations involving chromosomes 9, 10, and 14 (M6 and M7).
fects of infectious agents (mycoplasmas) that are naturally encountered. In this study, we showed that infections by several human mycoplasmas prevented apoptosis and induced continued proliferation of 32D cells in culture without IL-3 supplement. We believe this is the first reported finding that infection by a prokaryote agent replaces the action of a growth factor to which the targeted cells normally respond. Interaction with a mycosplasmal membrane component(s) on the cell surface transmitted a signal(s) that had potent antiapoptotic effects and rescued 32D cells from cell cycle arrest. This mycoplasma-mediated growth-signaling pathway was apparently different from that of IL-3 in supporting continued growth of 32D cells. Activation of previously inactive NF-κB in 32D cells by the mycoplasmas appeared to be closely associated with their ability to rescue these cells from apoptosis in culture deprived of IL-3. Infections by mycoplasmas that markedly enhanced AP-1 activity but did not activate NF-κB failed to support growth of 32D cells in IL-3-free culture. Moreover, blocking activation of NF-κB by an inhibitor led to prominent cell death of 32D cells that were otherwise induced to grow by mycoplasmas. This finding is consistent with recent reports from several laboratories showing that NF-κB appears to mediate survival signals that protect cells from dying of apoptosis (2, 31, 33).

It became clear that rapid activation of NF-κB and induction of continued cell growth in 32D cells did not require infections by live mycoplasmas. Heat-killed mycoplasmas or mycosplasmal membrane preparation LAMPs could effectively activate NF-κB and induce continued growth of 32D cells in IL-3 free culture. The active component that triggered the signaling of NF-κB activation is most likely the lipid moiety of mycosplasmal membrane lipopeptides (8, 9, 25, 26). By continually supplying heat-killed mycoplasmas, we could maintain 32D cells in culture without IL-3 supplement for at least 2 months. Growth of these 32D cells remained dependent on the presence of a mycoplasma-mediated growth signaling(s) for survival. They quickly began to die of apoptosis when transferred to culture without supplement of IL-3 or heat-killed mycoplasmas.

In comparison, infections by live mycoplasmas not only rescued 32D cells from cell cycle arrest and supported continued cell growth in IL-3-free culture but also induced malignant transformation of 32D cells. However, similar to our earlier finding for C3H cells (30), the mycoplasma-mediated cell transformation process would take time and involve a period of latency. In this study, it took more than 4 to 5 weeks of chronic infection by M. fermentans or M. penetrans before some of the 32D cells with autonomous growth ability began to emerge. Initially, the 32D cells that had acquired the unregulated growth property and no longer required the support from IL-3 or mycoplasmas constituted apparently only a small population. Further prolonged infection by the live mycoplasmas in culture could produce more cells with the malignant ability of autonomous growth. Antibiotic eradication of mycoplasmas from these IL-3-free cultures infected by the mycoplasmas effectively selected for a population(s) or clones of transformed cells that were capable of continued growth without the support of growth signaling from either IL-3 or mycoplasmas (Fig. 6). These transformed 32D cells were highly tumorigenic when injected into animals. It is not known whether infections by all mycoplasmas capable of supporting continued growth of 32D cells in IL-3-free culture could subsequently transform 32D cells.

Interestingly, the transformed 32D cells that obtained the malignant property of unregulated growth induced by chronic mycoplasmal infection showed no evidence of NF-κB activation found in the early stage of mycoplasmal infection (Table 1). Infection by the mycoplasmas for 4 to 5 weeks apparently had irreversibly activated an oncogenic process, not involving NF-κB, that was constantly signaling growth to the 32D cells. Previous studies by others and by us showed chronic mycoplasmal infections produced chromosomal instability in mammalian cells (24, 30). Karyotypic analysis revealed development of unregulated cell growth ability and tumorigenic properties of 32D cells following infection by either M. fermentans or M. penetrans was associated with chromosomal changes and trisomy 19. Infection by M. penetrans appeared to be particularly effective in causing trisomy 19 and hence malignant transformation of 32D cells in IL-3-free culture. A great majority (95%) of cells were found to have trisomy 19 after 4 to 5 weeks of M. penetrans infection, without clonal selection by ciprofloxacin treatment (Table 2). Growth of these cells had apparently become unregulated, since they formed tumors in two of three injected nude mice.

Extensive cell cycle studies in recent years helped the development of important concepts of checkpoints and rate-limiting steps in the cell cycle (22). Because cancer is largely a somatic genetic disease, loss of the ability to effectively coordinate the progression of cell cycle events when damage prejudicial to cell division has occurred often leads to development of malignancy. Although the molecular mechanism of mycoplasmal-mediated oncogenesis is still not clear, our present study showed infections by M. fermentans and M. penetrans caused infidelity of genomic transmission in cell division. There were apparent aberrations in the machinery of chromosomal segregation as well as cell cycle checkpoint controls in mycoplasmal-infected 32D cells. Following a few weeks of infections by either of the mycoplasmas, 32D cells with chromosomal changes and trisomy 19 began to appear and gradually accumulated in culture. It is not known how obtaining additional chromosome 19 was associated with better autonomous growth ability of 32D cells in culture without IL-3 support. Study of overexpression or constitutive activation of various oncogenes in the mycoplasmal-transformed 32D cells is in progress.

In a previous study using monolayer culture of murine C3H embryonic cells, we showed that mycoplasmas induced malignant transformation of mammalian cells through chronic persistent infection. The process appeared to be a gradual progression with multiple distinct stages characterized by reversibility or irreversibility of transformation (30). In the present model system, using suspension culture of murine hematopoietic cells, we elucidated two separable avenues of mycoplasmal effects on mammalian cells. The first avenue rapidly activated an antiapoptotic cascade of events exerted through membrane component(s), rescued cells from cell cycle arrest, and induced continued cell growth. The mitogenic effect of mycoplasmal-mediated signaling was reversible. The second avenue required infection by live organisms with a latent period, later causing infidelity of genomic transmission in cell division resulting in malignant cell transformation. The effect of the first avenue to induce continued proliferation of cells that would otherwise undergo apoptosis was a prerequisite for subsequent induction of irreversible cell transformation associated with chromosomal changes. Since the mycoplasmamediated transformation did not require mycoplasmal DNA integration into the host cells (37) or continued presence of the microbes once the genetic changes that lead to unregulated cell growth occurred, it presented a unique form of “hit and run” process.

Finding that human mycoplasmas can render growth factor independence and induce malignant transformation of IL-3-dependent hematopoietic cells in vitro has not only significance in general biology but also great direct clinical impli-
cations. In addition to further understanding the molecular mechanisms of mycoplasma-mediated pathway(s) for growth factor independence and oncogenesis, some important questions need to be answered. Can mycoplasmal infections also induce malignant transformation of human blood cells in culture? Is it possible to develop an animal model for mycoplasma-induced malignancies? These important studies will prove to be highly challenging due to the chronic nature of mycoplasmal infections and the long latency in oncogenesis in vivo. However, resolving these pieces of the puzzle could fundamentally change the way in which we view many human malignancies.

ACKNOWLEDGMENTS

We thank Douglas J. Wear for critical reading of the manuscript. We also thank Mark Tsai for assistance with Storm600 scanner and Susan Ditty for help with preparation of the manuscript.

This work was supported in part by the American Registry of Pathology.

REFERENCES

1. Barile, M. F., G. P. Bodey, J. Snyder, D. B. Riggs, and M. W. Grabowski. 1966. Isolation of Mycoplasma orale from leukemic bone marrow and blood by direct culture. J. Natl. Cancer Inst. 36:155–159.

2. Beg, A. A., and D. Baltimore. 1996. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 274:782–784.

3. Blaser, M. J., and J. Parsonnet. 1994. Parasitism by the “slow” bacterium Helicobacter pylori leads to altered gastric homeostasis and neoplasia. J. Clin. Invest. 94:4–8.

4. Cover, T. L., and M. J. Blaser. 1998. A Myco-

5. Garcia, J., B. Lemercier, S. Roman-Roman, and G. Rawadi. 1992. Kinetic analysis of cytokine gene expression in the livers of naive and immune mice infected with Listeria monocytogenes. Proc. Natl. Acad. Sci. USA 89:1103–1106.

6. Feng, S.-H., and S.-C. Lo. 1995. Mycoplasmas and oncogenesis: persistent infection and multistage malignant transformation. Proc. Natl. Acad. Sci. USA 92:10197–10201.

7. Greenberger, J. S., R. J. Eckner, M. Sakakeeny, P. Marks, D. Reid, G. Nabel, A. Hapel, J. N. Ihle, and K. C. Humphries. 1993. Interleukin 3-dependent hematopoietic progenitor cell lines. Fed. Proc. 42:2762–2771.

8. Greenberger, J. S., M. A. Sakakeeny, R. K. Humphries, C. J. Eaves, and R. J. Eckner. 1983. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc. Natl. Acad. Sci. USA 80:2931–2935.

9. Hayflick, L., and H. Koprowski. 1965. Direct agar isolation of mycoplasmas from human leukemic bone marrow. Nature (London) 205:713–714.

10. Lo, S. C., M. M. Hayes, R. Y. Wang, P. F. Pierce, H. Kotani, and J. W. Shih. 1991. Newly discovered mycoplasma isolated from patients infected with HIV. Lancet 338:1415–1418.

11. Loo, V. G., S. Richardson, and P. Quinn. 1991. Isolation of Mycoplasma pneumoniae from pleural fluid. Diagn. Microbiol. Infect. Dis. 14:443–445.

12. Murphy, W. H., C. Bullis, L. Dabich, R. Heyn, and C. J. Zarafonetis. 1970. Isolation of mycoplasma from leukemic and nonleukemic patients. J. Natl. Cancer Inst. 45:243–251.

13. Murphy, W. H., C. Bullis, I. J. Ertel, and C. J. Zarafonetis. 1967. Mycoplasma studies of human leukemia. Ann. N. Y. Acad. Sci. 143:544–556.

14. Murphy, W. H., D. Furtado, and E. Plata. 1965. Possible association between leukemia in children and virus-like agents. JAMA 191:110–115.

15. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warner, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. Helicobacter pylori infection and gastric lymphoma. N. Engl. J. Med. 333:1267–1271.

16. Ratner, G. J., P. Jacobs, and F. T. Perkins. 1965. Chromosome changes in human diploid-cell cultures infected with Mycoplasma. Nature 207:43–43.

17. Rawadi, G., J. Garcia, B. Lemercier, and S. Roman-Roman. 1999. Signal transduction pathways involved in the activation of NF-kappaB, AP-1, and c-fos by Mycoplasma fermentans membrane lipoproteins in macrophages. J. Immunol. 162:2193–2203.

18. Sach, G., A. Märtens, U. Deiters, R. Süßmuth, G. Jung, E. Wingender, and P. F. Mulholland. 1998. Activation of nuclear factor-xB in macrophages by mycoplasmal lipopolysaccharide. Eur. J. Immunol. 28:207–212.

19. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with ‘mini-extracts’, prepared from a small number of cells. Nucleic Acids Res. 17:6419.

20. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Detection of octamer binding proteins with ‘mini-extracts’, prepared from a small number of cells. Nucleic Acids Res. 17:6419.

21. Simbeck, J. W., J. K. Davis, M. K. Davidson, S. E. Ross, C. T. K.-H. Stadtlander, and G. H. Cassentein. 1998. Mycoplasma diseases of animals. p. 391–416. In J. B. Benson, L. R. Finch, J. Maniloff, and R. N. McElhaney (ed.), Mycoplasmas: molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.

22. Taylor-Robinson, D. 1989. Genetic mycoplasma infections. Clin. Lab. Med. 9:501–523.

23. Tsai, S., D. J. Wear, J. W. Shih, and S. C. Lo. 1995. Mycoplasmas and oncogenesis: persistent infection and multistage malignant transformation. Proc. Natl. Acad. Sci. USA 92:10197–10201.

24. Van Antwerp, D., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma. 1999. Signal transduction pathways involved in the activation of NF-kappaB, AP-1, and c-fos by Mycoplasma fermentans membrane lipoproteins in macrophages. J. Immunol. 162:2193–2203.

25. Vincenti, M. P., T. A. Burrell, and S. M. Taffet. 1992. Regulation of NF-

26. Wang, R. Y.-H., and S.-C. Lo. 1995. Mycoplasma and oncogenesis: persistent infection and multistage malignant transformation. Proc. Natl. Acad. Sci. USA 92:10197–10201.

27. Wang, R. Y.-H., and S.-C. Lo. 1993. PCR detection of Mycoplasma fermentans infection in blood and urine. J. Infect. Dis. 168:1668–1683.

28. Wang, R. Y.-H., and S.-C. Lo. 1993. PCR detection of Mycoplasma fermentans infection in blood and urine. J. Infect. Dis. 168:1668–1683.

29. Zhang, B., S. Tsai, J. W.-K. Shih, D. J. Wear, and S.-C. Lo. 1998. Absence of mycoplasmal gene in malignant mammalian cells transformed by chronic persistent infection of mycoplasmas. Proc. Soc. Exp. Biol. Med. 218:82–88.