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Invasion of Vero Cells and Induction of Apoptosis in Macrophages by Pathogenic *Leptospira interrogans* Are Correlated with Virulence

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Interactions of virulent *Leptospira interrogans* serovar icterohaemorrhagiae strain Verdun with Vero cells (African green monkey kidney fibroblasts) and a monocyte-macrophage-like cell line (J774A.1) were assayed by a double-fluorescence immunolabelling method. Infectivity profiles were investigated according to (i) the duration of contact between leptospires and eukaryotic cells and (ii) the number of in vitro passages after primary isolation from lethally infected guinea pigs. Comparative experiments were conducted with the corresponding high-passage avirulent variant and the saprophytic leptospire *Leptospira biflexa* Patoc I. In Vero cells, virulent leptospires were quickly internalized from 20 min postinfection, whereas avirulent and saprophytic strains remained extracellularly located. In addition, the virulent strain demonstrated an ability to actively invade the monocyte-macrophage-like J774A.1 cells during the early stages of contact and to induce programmed cell death, as shown by the detection of oligonucleosomes in a quantitative sandwich enzyme immunoassay. In both cellular systems, subsequent in vitro subcultures demonstrated a progressive decrease of the invasiveness, pointing out the necessity of using primocultures of *Leptospira* for virulence studies. Invasiveness of virulent leptospires was significantly inhibited with monodansylcadaverine, indicating that internalization was dependent on receptor-mediated endocytosis. Invasion of epithelial cells and induction of apoptosis in macrophages may be related to the pathogenicity of *Leptospira*, and both could contribute to its ability to survive in the host and to escape from the immune response.

Leptospirosis is a worldwide zooanthroponosis caused by bacteria belonging to the genus *Leptospira* (14), which includes classically two species, *Leptospira interrogans*, pathogenic for humans and animals, and *Leptospira biflexa*, a saprophyte found in surface waters and soils. Traditionally, each *Leptospira* species was divided into taxa, called serovars, by a classification based on a pairwise comparison of the extent of cross-absorption of antisera raised against each serotype (27). Antigenically related serovars constitute serogroups: *L. interrogans* includes 202 serovars distributed in 23 serogroups; *L. biflexa* includes 65 serovars in 38 serogroups (27). Molecular methods have given a new insight into *Leptospira* taxonomy. On the basis of DNA relatedness, seven pathogenic species and two saprophytic species have been previously described (39, 45). rRNA-gene restriction patterns (36) and PCR-derived methods (37) have confirmed this diversity within each genomic species and in some serovars of epidemiological importance. The wide spectrum of clinical symptoms, including fever, myalgia, meningitis, jaundice, renal failure, and ocular disorders, makes the diagnosis of human leptospirosis difficult (35, 44), and the course of the disease varies from mild to rapidly fatal forms (14). The most severe human diseases are caused by *Leptospira interrogans* serovar icterohaemorrhagiae (14), which is found worldwide and groups isolates closely related in a clonal mode (19).

Our knowledge of the physiopathology of leptospirosis remains very limited. Adhesion to cell surfaces and toxicity seem to be important properties of virulent leptospires in pathogenesis (14), but these phenomena are poorly documented. Virulent leptospires reach the target tissues of the host organism by a bacteremic stage and can colonize privileged sites where the immune system is less efficient, such as proximal renal tubules (12) and aqueous ocular humor (1). Avirulent leptospires are cleared rapidly, within a few minutes of entry, by reticuloendothelial phagocytosis (13). Virulent leptospires which survive by evading phagocytosis can be isolated ordinarily from the blood in the first week of the course of the disease, but late isolation of leptospires in blood (40) or in cerebrospinal fluid (CSF) (32) has also been reported. Saprophytic strains were isolated from the environment but never from a living organism since they are unable to develop pathogenicity on passage through an animal body (14).

Little is known concerning virulence factors in *Leptospira*. Pathogenic leptospires are cultured in media not resembling conditions in vivo, and virulence is gradually lost after some passages (14). This phenotypic switch takes place during serial culture in vitro and is hard to relate to the 50% lethal dose, which is not an objectively measurable parameter of loss of virulence, while death is not an all-or-none measure of leptospirosis infection. Virulence can be restored before the irreversible loss by inoculating susceptible animals such as young guinea pigs (14). Hemolysins such as sphingomyelinase C have been previously purified (6) and the corresponding genes have been sequenced (41), but their involvement in pathogenesis is still unclear, even though chemotaxis of pathogenic leptospires to hemoglobin was recently described (46). Attachment of *Leptospira* to eukaryotic cells, suggesting a relationship between virulence and adhesion in pathogenic leptospires, has been investigated in L929 fibroblastic cells (43) and renal epithelial cells (3). Finally, an invasion assay revealed that pathogenic *Leptospira* can enter the MDCK and human umbilical

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vein epithelial cell cytoplasm (42). Adhesion to components of the extracellular matrix is thought to occur for *Leptospira* (22, 23). However, comparative studies between virulent and avirulent strains belonging to the same serovar are limited and the mechanisms of host cell entry used by leptospires are not elucidated. Leptospiral lipopolysaccharide was able to induce secretion of interleukin-1 and production of interferon by macrophages, but leptospiral lipopolysaccharide preparations extracted from strains differing in virulence did not enhance significant variations in the activation of macrophages (21). A previous study on the interaction between macrophages and leptospires (8) suggested that macrophages could not be considered an efficient defense against infective leptospires, but no mechanism to explain the escape of virulent leptospires to nonspecific immune response was proposed.

In this report, we investigate the interactions of a virulent strain from *L. interrogans* serovar icterohaemorrhagiae, its corresponding high-passage-number avirulent variant, and the saprophytic leptospire *L. biflexa* Patoc I with relevant culture cell types related to the different stages of leptospirosis. Infection of renal epithelial cells (Vero) and the monocyte-macrophage-like cell line (J774A.1) were assayed by a double-fluorescence immunolabelling method (17). According to the duration of contact between leptospires and eukaryotic cells and the number of in vitro passages after primary isolation, infectivity profiles including cytoadherence and invasiveness were examined. In addition, the induction of programmed cell death of eukaryotic cells by pathogenic leptospires was investigated by an immunoassay detecting histone-associated DNA fragments.

MATERIALS AND METHODS

Bacterial strains and cultivation. Leptospires were grown in liquid EMJH medium (11) at 30°C under aerobic conditions to a density of about 10^8 bacteria per ml and counted in a Petroff-Hausser counting chamber. The virulent *L. interrogans* Verdun (38), serovar icterohaemorrhagiae, was from the Reference Collection of the Pasteur Institute in Paris, France. From the virulent isolate Verdun, first isolated from a human during World War I in 1917, two isogenic clones were derived. One was maintained as virulent by deep-freeze preservation and iterative passage in guinea pigs. The other clone was regularly in vitro passaged to obtain the avirulent variant. This corresponding high-passage-number avirulent variant and the saprophytic strain Patoc I (*L. biflexa* serovar patoc) were grown under the same conditions as the virulent one. In invasion assays, both live and formalin-killed bacteria were used. Leptospires were killed after an incubation of 30 min at room temperature in phosphate-buffered saline (PBS), pH 7.2, containing 10% (vol/vol) formaldehyde solution (Merck, Darmstadt, Germany), washed three times in PBS, and resuspended in cell culture medium.

Specific-pathogen-free Dunkin-Hartley ICO:DH (Poc) guinea pigs were purchased from IFFA-CREDO, L'Arbresle, France, and bred at the Institute Pasteur, Nouméa, New Caledonia. Six guinea pigs 10 to 12 days old (weighing less than 150 g each) were lethally infected intraperitoneally with 10^8 leptospires of a virulent culture in EMJH medium. Another group of three animals was inoculated by the same route with sterile EMJH medium and used as a control. When prostration, anorexia, and jaundice appeared between 4 and 6 days after inoculation, blood from three infected guinea pigs was collected by cardiac puncture and cultured in EMJH medium (this highly virulent clone was named for passage 0 [P0]). The three remaining animals were kept until death as lethality controls. P0 was regularly passaged between 7 and 10 days of culture (at least 10^8 leptospires per ml) in EMJH medium so that the virulence was progressively decreased by passage 6.

Immunological reagents. New Zealand White rabbits, weighing approximately 3 to 4 kg each, were used for the immunizations with live *Leptospira* strains according to reference methods (44). The antibody titer was determined by means of the microscopic agglutination test (20, 28), and titers of at least 1:12,800 were obtained. Sera were aliquoted and stored at −20°C until use.

Cell cultures. The murine monocyte-macrophage-like J774A.1 and the Vero (African green monkey kidney fibroblast) cell lines were maintained in RPMI 1640 and in modified Eagle medium (MEM) (Sigma Chemical Co., St. Louis, Mo.), respectively. Both media were buffered with sodium bicarbonate and supplemented with 10% fetal calf serum, 5 mM L-glutamine, 100 μg of ampicillin per ml, and 100 μg of streptomycin (Sigma Chemical Co.) per ml. The cells were cultured in an atmosphere containing 5% CO_2 at 37°C.

Invasion procedure. J774A.1 cells were scraped and washed in RPMI 1640 medium without antibiotics. Cell viability was determined by the trypan blue exclusion test, and cell suspensions (1 ml of 10^5 cells per ml) were layered over round glass coverslips in 24-well tissue culture plates (cluster plates; Costar Cambridge, Mass.).

Vero cells were harvested with 0.05% trypsin and 0.02% EDTA in a balanced salt solution (Flow Laboratories, Seven Hills, New South Wales, Australia) and plated in 24-well plates in MEM without antibiotics as described above. The plates were incubated for 48 h and washed twice with medium without antibiotics to remove nonadherent cells. Bacteria were harvested by centrifugation (12,000 × *g* for 15 min), washed twice in PBS, and then suspended in warm (37°C) cell culture medium without antibiotics to a concentration of 10^7 per ml. Pathogenic and saprophytic *Leptospira* strains were used at the same time in each experiment. A 1-ml aliquot of each bacterial suspension was then added to the corresponding wells (at a bacterium/cell ratio of 100:1), and the plates were incubated under static conditions for 2 h at 37°C. Each 20 min, the corresponding coverslips were recovered and washed twice in PBS to remove nonadherent bacteria. Experiments were performed in triplicate.

Double fluorescence staining. A 50-μl aliquot of the leptospiral rabbit antiserum diluted 1 to 100 in PBS containing 0.5% bovine serum albumin (BSA) was applied to the wet coverslips for 30 min at room temperature before being washed three times in PBS, fixed for 2 min in pure methanol at −20°C, and air dried, and the coverslips were adhered to glass slides with Eukitt (Kindler, Freiburg, Germany). The slides were incubated for 30 min at 37°C in a moist chamber with goat anti-rabbit immunoglobulin G fluorescein $F(ab')_2$ fragment (Boehringer, Mannheim, Germany) diluted 1 to 10 in PBS–0.5% BSA to stain extracellularly located bacteria. The coverslips were extensively washed by dipping into PBS and subjected to a second incubation with the same leptospiral rabbit antiserum under the same conditions. Then, intracellularly located bacteria were stained for 30 min at 37°C with goat anti-rabbit immunoglobulin G rhodamine $F(ab')_2$ fragment (Boehringer) diluted 1 to 10 in PBS–0.5% BSA. After excess antibody was washed off with PBS, slides were mounted in glycerol-PBS and examined with the Leitz DMRBE epifluorescence microscope with either a blue filter (excitation and emission wavelengths, 450 and 490 nm, respectively) for cell-attached *Leptospira* or a green filter (excitation and emission wavelengths, 515 and 560 nm, respectively) for intracellular *Leptospira*. Randomly selected cells in each of two coverslips at 20, 40, 60, 80, 100, and 120 min postinoculation (p.i.) were examined in order to count infected cells (with cell-attached and/or intracellular bacteria) and to determine the number of cell-attached and intracellular *Leptospira*. Internalization was expressed as the mean number of bacteria internalized per cell. Intracellular *Leptospira* organisms, identified as rhodamine-labelled bacteria not labelled with fluorescein, were quantitated by taking the difference between rhodamine- and fluorescein-labelled spirochetes. The mean was calculated by averaging the number of bacteria present in 100 cells in three independent assays, and the standard deviation was determined. Photomicrographs of representative cells were taken with ultra-high-speed (1600 ASA) reversed film (Eastman Kodak Company, Rochester, N.Y.) by using an Orthomat E system.

Inhibitory biochemicals. Stock solutions of compounds (all from Sigma Chemical Co.) were diluted in dimethyl sulfoxide as indicated below and stored at −20°C. The inhibitors of microtubule system formation and receptor-mediated endocytosis were nocadazole (3.3 mM) and monodansylcadaverine (30 mM), respectively. Cytochalasin D (2 mM) was used as an inhibitor of actin microfilament polymerization. To obtain working concentrations, stock solutions were diluted with either RPMI 1640 or MEM. Working concentrations were 0.1, 1, and 10 μM for nocadazole; 1, 10, and 100 μM for monodansylcadaverine; and 0.02, 0.2, and 2 μM for cytochalasin D. Cells were pretreated for 1 h before the addition of bacteria and throughout the invasion assays. Treatment of the bacteria and eukaryotic cells with the different compounds at the upper concentrations did not significantly reduce cell or bacterium viability (data not shown).

Enzyme immunoassay for measurement of apoptosis. The cell death detection enzyme-linked immunosorbent assay (Boehringer), a quantitative sandwich enzyme immunoassay with mouse monoclonal antibodies directed against DNA and histones, was used. This assay provides a qualitative and quantitative determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates and allows the monitoring of the time course of DNA fragmentation during apoptosis in many different cell systems. Briefly, cells were diluted with culture medium without antibiotics to obtain a suitable cell concentration (10^5 cells per ml) and transferred into Eppendorf tubes (500 μl of 5×10^4 cells per ml) in duplicate. Thereafter, 500 μl of each bacterial suspension (10^7 per ml) in cell culture medium was added to corresponding tubes. Samples were incubated under static conditions for 5 h at 37°C and processed each hour. According to the manufacturer's instructions, target cells were disrupted and the fragmented DNA was collected after centrifugation of the cytoplasmic fraction. Detection of the anti-DNA-peroxidase conjugate was performed with ABTS [2,2′-azino-di-(3-ethylbenzthiazolinesulfonic acid)] as substrate and quantitated photometrically at 405 nm (Titertek Multiskan). Samples without *Leptospira* cells were used as negative controls. The results reported are mean values of two independent assays ± standard deviations.

RESULTS

We applied the double-fluorescence immunolabelling method to discriminate between intracellular and extracellular

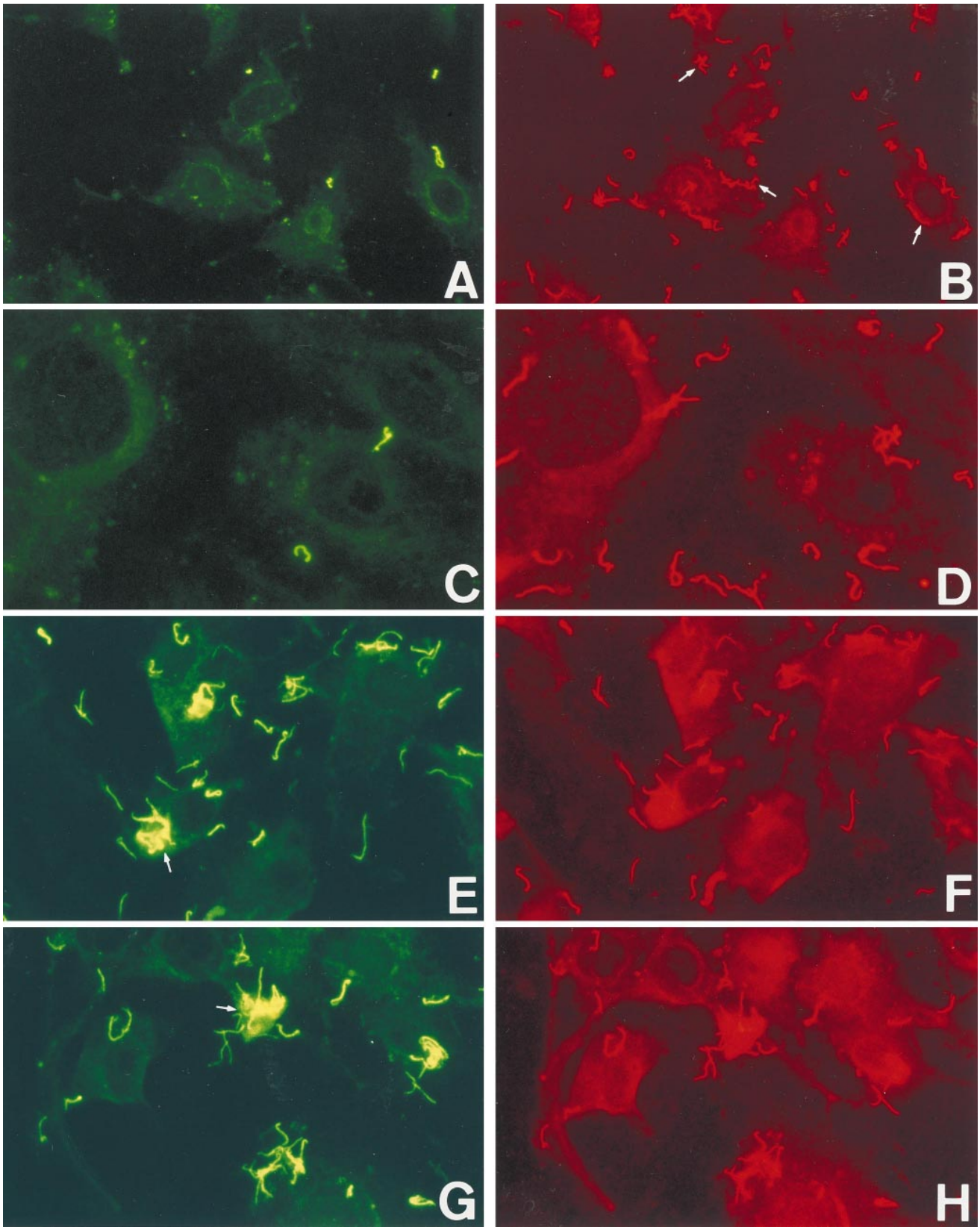

FIG. 1. Dual fluorescent staining of leptospires in infected Vero cells and visualization with FITC or TRITC filters (see Materials and Methods). (A) Virulent variant of strain Verdun (*L. interrogans* serovar icterohaemorrhagiae). Vero cells with a small number of green extracellular leptospires are shown. (B) Same field as that in panel A but showing numerous additional red internalized bacteria (arrows). (C and D) Same experiment as that in panel B but at a higher magnification. Note the typical morphology of the leptospires. (E and F) Avirulent variant of strain Verdun. Clumps of extracellular leptospires are indicated (arrow [E]). Note the absence of additional leptospires seen with the red filter. (G) Saprophytic strain Patoc I (*L. biflexa* serovar patoc). Clumps of extracellular leptospires are indicated (arrow). (H) Same field as that in panel G with the red filter without additional bacteria. Magnification, ×360 (A and B), ×720 (C and D), and ×750 (E to H).

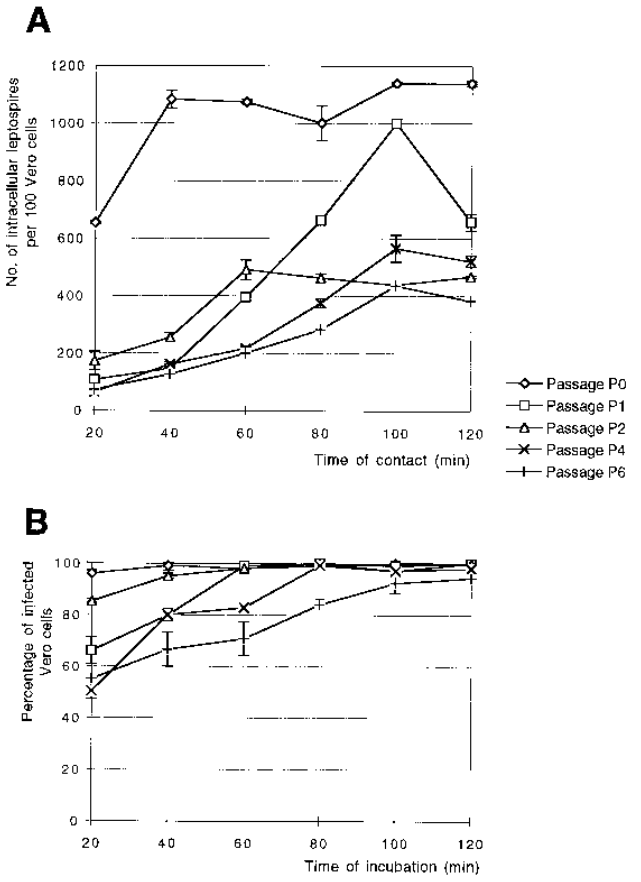

FIG. 2. Kinetics of the internalization of virulent *L. interrogans* serovar icterohaemorrhagiae in Vero cells (A) and the percentages of infected cells (B) according to the number of in vitro passages of the virulent strain and the incubation time (as defined in Materials and Methods). Results are the means of triplicate experiments ± standard errors (bars).

Leptospira bacteria in the same microscopic field. This staining procedure has been applied to nonprofessional as well as professional phagocytic cell lines (17). The cell-associated leptospires were distributed among cell-attached or extracellular bacteria (green and red fluorescence with the fluorescein isothiocyanate [FITC] and tetramethyl rhodamine isothiocyanate [TRITC] filter systems, respectively) and intracellular bacteria (additional red fluorescence after TRITC filtering).

Time course of interaction of leptospires with Vero cells. (i) Kinetic studies with virulent *Leptospira*. Virulent leptospires were mainly intracellularly located (>90%) in Vero cells. Intracellular localization was evident from 20 min p.i., and internalized virulent leptospires with their typical morphology were isolated throughout the cytoplasm (Fig. 1). The percentages of infected cells, containing at least one extra- or intracellularly located bacterium, were easily quantitated for the primoculture and its subsequent subcultures. Typical results (ratio of infected cells to total screened cells) were expressed as mean percentages ± standard deviations (Fig. 2B). The cell association of the primoculture (P0) in 2-day-old Vero cell monolayers showed a high percentage (96.0 ± 2.0%) of infected cells from 20 min p.i. According to the passage (P1 to P6), this percentage decreased in the first sampling time (20 min) but was always >90% in the late sampling time (120 min), exhibiting a time-dependent infectivity profile with saturation over a 2-h period.

Concurrently, the infectivity profile exhibited a progressive increase of the number of intracellular leptospires per 100 Vero cells, with a maximum reached in less than 2 h depending on the passage (Fig. 2A). A loss of invasiveness was distinctly observed with the subsequent subcultures of the virulent strain, markedly between P0 and P1. As an example, 1,137 ± 9, 656 ± 29, 466 ± 4, 520 ± 20, and 382 ± 6 internalized bacteria/100 Vero cells were counted 120 min p.i. for P0, P1, P2, P4, and P6, respectively. However, the number of extracellular cell-associated virulent leptospires never increased significantly (data not shown).

(ii) Kinetic studies with avirulent *Leptospira*. Interactions of the avirulent Verdun strain with Vero cells were compared with those of the virulent strain. Conversely to virulent *Leptospira*, the avirulent variant was solely located extracellularly, as shown in Table 1. No additional bacteria were visible with the TRITC filter system (Fig. 1). The infectivity profile was time dependent, with a maximum value of infected cells of 100% reached at 60 min p.i. At the sampling times of 100 and 120 min p.i., corresponding to the end of the 2-h incubation period, the number of cell-associated leptospires was quantitated with less accuracy. Indeed, adherent avirulent leptospires were clustered in clumps of 10 to 20 organisms like balls of wool (Fig. 1). The interaction of the avirulent *Leptospira* strain with Vero cells gave the maximum number of cell-associated leptospires, with at least 2,185 ± 85 bacteria per 100 cells (120 min p.i.) in this in vitro model.

(iii) Kinetic studies with saprophytic *Leptospira*. As shown in Table 1, the cell-associated saprophytic leptospires were never encountered intracellularly whatever the length of the incubation period. The percentage of infected cells increased gradually in a time-dependent manner from 68.3 ± 9.7% (20 min p.i.) to a maximum value of 91.0 ± 1.0% (100 min p.i.). Concurrently, the number of cell-associated saprophytic leptospires per 100 Vero cells increased from 250 ± 54 (20 min p.i.) to 878 ± 32 (120 min p.i.), showing a saturation of Vero cell sites available for leptospire anchorage in the final sampling times. Numerous saprophytic leptospires adhered to areas of the glass coverslips not covered by epithelial renal cells.

Time course of interaction of leptospires with macrophages. (i) Kinetic studies with virulent *Leptospira*. Qualitatively, virulent leptospires were located intracellularly in a high proportion (>90%) in J774A.1 cells from 20 min p.i. whatever the passage. Intracellular virulent *Leptospira* was mainly isolated in the cytoplasm or in small clusters of two to three organisms with its typical morphology (Fig. 3). At late sampling times, some of them were more abundant at the periphery of the nucleus. The number of infected cells was quantitated by scanning the coverslips at a low magnification. Typical results are presented in Fig. 4B. J774A.1 cells are readily infected with the virulent *Leptospira* strain in a time-dependent manner. Whatever the passage, the ratio of infected cells increased progressively to reach a maximum between 60 and 80 min p.i. However, at each 20-min step, the ratio of infected cells regularly decreased with the number of passages. This phenomenon was markedly visible with J774A.1 cells throughout the infection experiments. As an example, 79.5 ± 1.6, 47.6 ± 2.4, 43.0 ± 5.0, 33.9 ± 1.8, and 27.2 ± 1.7% of J774A.1 cells were infected for P0, P1, P2, P4, and P6, respectively, after 20 min of contact at 37°C.

The number of internalized leptospires per 100 cells followed approximately the same time-dependent pattern as shown in Fig. 4A. The infectivity profile exhibited a gradual increase whatever the passage, with a maximum reached in less than 2 h p.i. (575 ± 33 intracellular bacteria/100 J774A.1 cells at 120 min p.i. for P0). Similarly, at each 20-min step, the

TABLE 1. Distribution of virulent (P0) and avirulent variants of *L. interrogans* serovar icterohaemorrhagiae strain Verdun and saprophytic strain Patoc I (*L. biflexa* serovar patoc) during infection[a]

Variant	Length of incubation (min)	% Infected Vero cells	No. of cell-associated leptospires/100 Vero cells	% Internalized leptospires	% Infected J774A.1 cells	No. of cell-associated leptospires/100 J774A.1 cells	% Internalized leptospires
Virulent P0	20	96.0 ± 2.0	659 ± 3	99.1 ± 0.3	79.5 ± 1.6	176 ± 2	100 ± 0.0
	40	99.0 ± 1.0	1,168 ± 18	92.7 ± 1.2	85.0 ± 1.8	310 ± 3	95.8 ± 2.0
	60	98.0 ± 2.0	1,106 ± 0	97.1 ± 0.7	97.0 ± 3.0	337 ± 8	98.2 ± 1.5
	80	99.0 ± 1.0	1,000 ± 60	100 ± 0.0	96.2 ± 1.6	456 ± 33	97.4 ± 2.2
	100	100.0 ± 0.0	1,150 ± 4	99.0 ± 0.1	100.0 ± 0.0	551 ± 11	99.5 ± 0.1
	120	100.0 ± 0.0	1,156 ± 8	98.3 ± 0.1	97.1 ± 2.9	575 ± 33	99.6 ± 0.4
Avirulent	20	85.0 ± 1.0	213 ± 1	0	68.8 ± 2.0	233 ± 6	0
	40	96.2 ± 2.0	468 ± 28	0	86.4 ± 2.0	280 ± 10	0
	60	100 ± 0.0	570 ± 62	0	98.1 ± 2.0	631 ± 9	5.5 ± 0.1
	80	100 ± 0.0	1,432 ± 58	0	100 ± 0.0	766 ± 12	10.2 ± 0.3
	100	100 ± 0.0	2,137 ± 117	0	100 ± 0.0	966 ± 0	12.0 ± 0.6
	120	100 ± 0.0	2,185 ± 85	0	100 ± 0.0	1,036 ± 4	13.4 ± 0.2
Saprophytic	20	68.3 ± 9.7	250 ± 54	0	88.2 ± 1.8	264 ± 7	0
	40	90.4 ± 3.6	343 ± 45	0	99.0 ± 1.1	432 ± 34	0
	60	89.5 ± 0.5	512 ± 24	0	99.2 ± 0.8	564 ± 26	2.8 ± 0.1
	80	89.5 ± 6.5	622 ± 68	0	100 ± 0.0	857 ± 16	7.0 ± 0.1
	100	91.0 ± 1.0	777 ± 65	0	100 ± 0.0	805 ± 7	6.8 ± 0.1
	120	88.0 ± 6.0	878 ± 32	0	100 ± 0.0	812 ± 4	6.6 ± 0.1

[a] Results are the means of triplicate experiments ± standard errors.

number of intracellular virulent *Leptospira* cells per 100 J774A.1 cells decreased with the progressive subculture of the strain. This loss of infectivity was distinctly observed between P0 and P1. As an example, 176 ± 2, 64 ± 4, 58 ± 8, 46 ± 6 and 28 ± 1 internalized leptospires/100 J774A.1 cells were counted 20 min p.i. for P0, P1, P2, P4, and P6, respectively.

(ii) Kinetic studies with avirulent *Leptospira*. In contrast to virulent *Leptospira*, the avirulent strain was mainly extracellularly located in J774A.1 cells, as shown in Table 1. The percentage of infected cells was determined at various times after inoculation and showed a time-dependent pattern, with a maximum of 100% reached from 80 min p.i. Internalized leptospires were only visible from 60 min p.i. and represented only a small proportion of the macrophage-associated bacteria. As an example, 5.5, 10.2, 12.0, and 13.4% of the cell-associated avirulent leptospires were located intracellularly at 60, 80, 100, and 120 min p.i., respectively, in J774A.1 cells; this could be considered as showing the basal phagocytosis activity. As shown in Table 1, the number of cell-associated leptospires increased with time during the course of the experiment until 100 min p.i., suggesting an approaching saturation of macrophage sites available for bacterial adherence. At the sampling times of 100 and 120 min p.i., corresponding to the end of the 2-h incubation period, the number of cell-associated leptospires was quantitated with less accuracy.

(iii) Kinetic studies with saprophytic *Leptospira*. The cell-associated saprophytic leptospires were mainly located extracellularly in J774A.1 cells. As shown in Table 1, intracellular leptospires were observed from 60 min p.i. Only 2.8, 7.0, 6.8, and 6.6% of the cell-associated saprophytic leptospires were internalized at 60, 80, 100, and 120 min p.i., suggesting a weak ability for internalization. The number of cell-associated leptospires increased gradually in a time-dependent manner to reach a plateau at 100 min p.i., showing a saturation of J774A.1 cell sites available for bacterial adhesion. Adherent saprophytic leptospires were clustered in clumps of 5 to 10 organisms, giving an undervaluation of the number of cell-associated bacteria at the last sampling times. In addition, numerous saprophytic leptospires adhered to areas of the glass coverslips not covered by eukaryotic cells.

Kinetics of attachment of formalin-killed leptospires in Vero and J774A.1 cells. To investigate whether active metabolism was required for adherence to eukaryotic cells, live and formalin-killed virulent (P0), avirulent, and saprophytic leptospires were compared in the adherence assay. As shown in Table 2, the time-dependent kinetics of cell association showed a saturation curve over a 2-h period but with a dramatically decreased percentage of infected J774A.1 and Vero cells. But more important is the absence of internalized formalin-killed virulent leptospires in Vero cells (Table 2) compared with live virulent leptospires (Table 1). These results suggested that internalization of virulent *L. interrogans* in those epithelial cells required a viable bacterium. The basal phagocytosis of J774A.1 cells could explain the small degree of internalization of formalin-killed virulent leptospires (Table 2).

Effects of inhibitors on internalization of virulent leptospires. Since most bacteria enter eukaryotic cells via a microfilament-dependent process, we studied the effects of cytochalasin D on the number of cell-associated leptospires and on the process of invasion. Cytochalasin D is an organic fungal compound which binds to actin, alters its polymerization, and in turn inhibits phagocytosis (10). As shown in Table 3, whatever the concentration used, cytochalasin D did not significantly reduced the percentage of infected cells and the internalization in both cell lines compared to the control without inhibitor. These results suggest that virulent leptospires did not enter via a microfilament-dependent pathway in our experimental model. Nocadazole reduced the invasive ability, as demonstrated in Table 3. A concentration of 10 μM decreased the number of infected J774A.1 cells by 30% and the number of internalized bacteria by 23%. The same concentration reduced the percentage of infected Vero cells by 20% and did not significantly decrease the internalization. Monodansylcadaverine decreased the number of internalized virulent *Leptospira* in

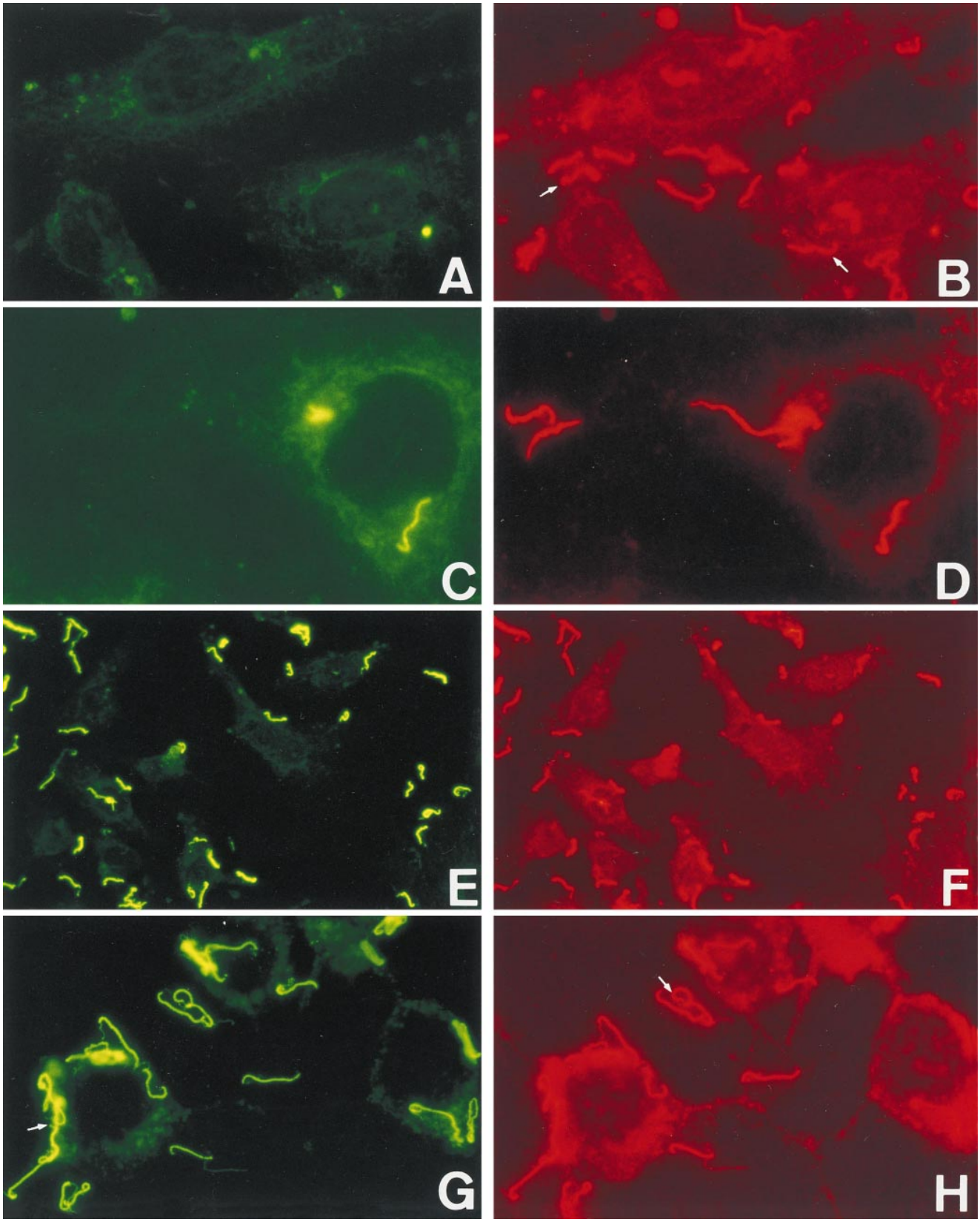

FIG. 3. Dual fluorescent staining of leptospires in infected J774A.1 cells and visualization with FITC or TRITC filters (see Materials and Methods). (A) Virulent variant of strain Verdun (*L. interrogans* serovar icterohaemorrhagiae). J774A.1 cells without extracellular leptospires are shown. (B) Same field as that in panel A but showing a great number of intracellular bacteria (arrows). (C and D) Same experiment as that in panel B but at a higher magnification. Note the typical morphology of the leptospires. (E and F) Avirulent variant of strain Verdun. Note the absence of additional leptospires with the red filter (F). (G and H) Saprophytic strain Patoc I (*L. biflexa* serovar patoc). Clumps of extracellular leptospires are indicated (arrow [G]). Note leptospires adhering to areas of the coverslips not covered by J774A.1 cells (H [arrow]). Magnification, ×900 (A, B, G, and H), ×1,130 (C and D), and ×450 (E and F).

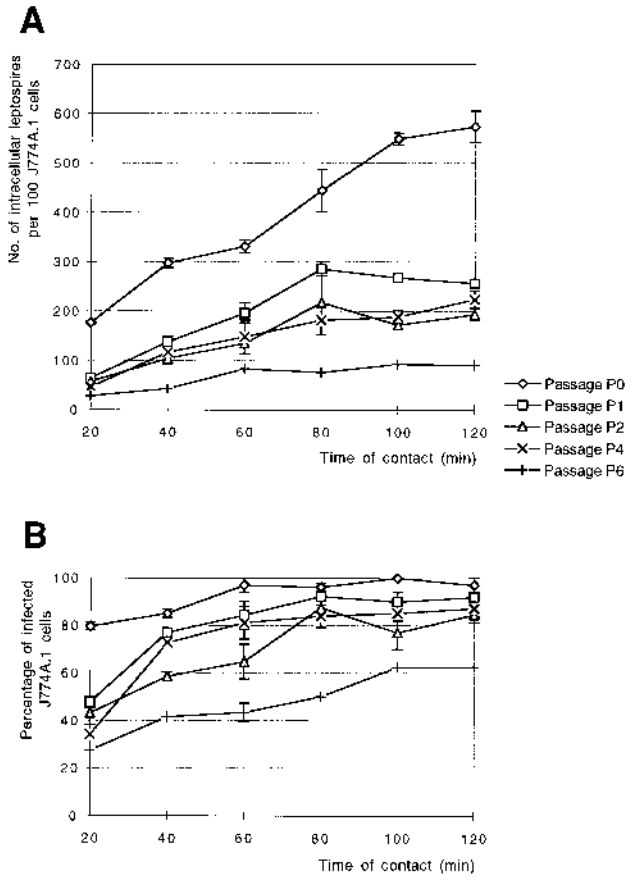

FIG. 4. Kinetics of the internalization of virulent *L. interrogans* serovar icterohaemorrhagiae in J774A.1 cells (A) and the percentages of infected cells (B) according to the number of in vitro passages of the virulent strain and the incubation time (as defined in Materials and Methods). Results are the means of triplicate experiments ± standard errors (bars).

a dose-dependent manner in J774A.1 and Vero cell lines but with different intensities (Table 3). A concentration of 100 μM decreased the internalization by 77% in J774A.1 cells; however, the same concentration significantly reduced the internalization by only 40% in Vero cells. Monodansylcadaverine also reduced the number of infected cells in a dose-dependent manner by 72% at a concentration of 100 μM but only in J774A.1 cells. In our studies, microtubules appeared to have a lesser role in leptospiral internalization than receptor-mediated endocytosis via coated pits.

Time course of DNA fragmentation. Enrichment factors, corresponding to the percentage of DNA fragmentation of the samples compared to the untreated control, were calculated from the set of absorbance values obtained at each point of the DNA fragmentation kinetic study. The internucleosomal degradation of genomic DNA occurring during apoptosis was evidenced only in J774A.1 cells infected with P0 of the virulent *Leptospira* strain after 2 h of contact (Fig. 5). Compared to respective control samples, the relative DNA fragmentation increased in a time-dependent manner to reach a maximum value of approximately 400% between 6 and 8 h of contact. However, no apoptosis occurred with P1 of the virulent strain (Fig. 5). Neither subsequent subcultures from P2 to P6 (data not shown), nor the avirulent variant, nor the saprophytic strain induced apoptosis. The presence of mono- and oligonucleosomes was not demonstrated when Vero cells were infected with the virulent P0 and its serial subcultures, the avirulent variant, or the saprophytic strain of *Leptospira* (data not shown).

DISCUSSION

Differentiation between extracellular and intracellular locations of bacteria is the most difficult problem in the microscopic evaluation of the invasiveness of a cell culture monolayer. Several methods are available to overcome this problem, and we used a double-fluorescence staining procedure which provided us with a useful, easy, and accurate tool to discriminate both intracellular and extracellular organisms within the same microscopic field. Our results suggest that virulent *L. interrogans* strains can readily invade eukaryotic host cells. However, the percentage of infected cells and the number of cell-associated virulent leptospires decrease with the progressive in vitro subcultures of the virulent strain, but leptospires are mainly located intracellularly. Many of the early studies investigated a limited number of cell lines that do not always represent those present during natural infections and were insufficiently quantitative to be conclusive. The virulence of the *Leptospira* strain tested was not systematically established or remained unclear (8, 22), or the bacterium/eukaryotic cell ratio was not available (3). In a previous study, Thomas and Higbie (42) showed in an in vitro model that specific attachment and invasion of endothelial and epithelial cells were properties of virulent leptospires. However, infectivity results were not correlated to progressive loss of virulence (leptospires at P5 or earlier were used) and were compared with only those from the saprophytic *L. biflexa* strain.

The data presented here demonstrate that monocyte-macrophage-like and Vero cell lines are permissive host cells for virulent leptospire invasion. The invasiveness was compared systematically with that of the corresponding extracellular avirulent and saprophytic strains showing a distinct profile of infection. In light of these results, virulent leptospires seem to behave like true invasive bacteria.

TABLE 2. Distribution of formalin-killed *Leptospira* (*L. interrogans* serovar icterohaemorrhagiae strain Verdun P0) during infection[a]

Length of incubation (min)	% Infected J774A.1 cells	No. of leptospires/100 J774A.1 cells			% Infected Vero cells	No. of leptospires/100 Vero cells		
		Cell associated	Intracellular	Extracellular		Cell associated	Intracellular	Extracellular
20	12.6 ± 0.7	13 ± 1	0	13 ± 1	15.7 ± 1.4	18 ± 1	0	18 ± 1
40	17.3 ± 1.8	21 ± 3	0	21 ± 3	18.4 ± 1.6	28 ± 2	0	28 ± 2
60	24.1 ± 2.7	30 ± 4	2 ± 1	28 ± 3	20.3 ± 1.7	34 ± 1	0	34 ± 1
80	27.9 ± 2.4	47 ± 2	3 ± 0	44 ± 2	26.5 ± 0.8	37 ± 2	0	37 ± 2
100	42.5 ± 1.6	55 ± 1	6 ± 1	49 ± 0	40.6 ± 0.2	57 ± 4	0	57 ± 4
120	36.3 ± 4.6	52 ± 1	5 ± 0	47 ± 1	41.6 ± 1.6	62 ± 2	0	62 ± 2

[a] Results are the means of triplicate experiments ± standard errors.

TABLE 3. Effects of inhibitors on the invasiveness of *L. interrogans* serovar icterohaemorrhagiae strain Verdun P0 of J774A.1 and Vero cells[a]

Eukaryotic cell line and inhibitor	Inhibitor concn (μM)[b]	% Infected cells	No. of intracellular leptospires/ 100 cells
J774A.1			
Cytochalasin D	0.00 (control)	72.0 ± 4.0	162 ± 26
	0.02	77.0 ± 2.0	167 ± 15
	0.20	79.0 ± 1.0	151 ± 22
	2.00	74.0 ± 5.0	173 ± 12
Nocadazole	0.0 (control)	86.2 ± 2.5	166 ± 15
	0.1	71.5 ± 1.7	152 ± 2
	1.0	60.0 ± 3.0	127 ± 4
	10.0	59.9 ± 2.0	127 ± 4
Monodansylcadaverine	0 (control)	72.0 ± 4.0	162 ± 26
	1	65.0 ± 5.0	122 ± 18
	10	49.0 ± 6.0	98 ± 29
	100	20.0 ± 2.0	38 ± 10
Vero			
Cytochalasin D	0.00 (control)	97.5 ± 0.5	793 ± 68
	0.02	93.0 ± 3.0	646 ± 79
	0.20	96.0 ± 2.0	606 ± 95
	2.00	96.0 ± 0.0	591 ± 79
Nocadazole	0.0 (control)	95.4 ± 0.5	782 ± 61
	0.1	91.2 ± 4.8	733 ± 22
	1.0	90.3 ± 2.4	696 ± 13
	10.0	76.0 ± 2.0	680 ± 32
Monodansylcadaverine	0 (control)	97.5 ± 0.5	793 ± 68
	1	96.0 ± 0.0	577 ± 79
	10	95.0 ± 5.0	391 ± 45
	100	96.0 ± 2.0	477 ± 31

[a] Results are the means of triplicate experiments ± standard errors.
[b] Reagents were added at 1 h preinfection, and infectivity was determined at 20 min postinfection.

Vinh et al. (43) and Thomas and Higbie (42) demonstrated the decrease of leptospiral adherence to host cells when leptospires were pretreated with formaldehyde; however, inhibition of internalization was not investigated. In our model, treatment with formaldehyde strongly inhibited internalization of virulent leptospires in both cell lines. This observation indicates that the viability or integrity of components of the leptospires is essential for the infectivity pattern. It is possible that the chemical treatment with formaldehyde altered or destroyed the integrity of the spirochetal ligand that initiates binding and internalization. Leptospires are very motile bacteria, and the formaldehyde treatment impaired their motility, thus the typical motility of *Leptospira* spp. might be considered a virulence factor (7). Leptospires have two periplasmic flagella between the outer membrane sheath and the cell cylinder (14); however, mutant strains lacking periplasmic flagella are not available for comparative invasiveness studies.

L. interrogans is sensitive to antibiotics currently used (33), and one hypothesis has proposed that the leptospires could have an intracellular phase in which they are protected from antibiotics and host immune system. Late isolations of leptospires from blood (40) or cerebrospinal fluid (32) have been described in previous studies. In an evaluation of PCR for the diagnosis of human leptospirosis (30), we reported an average persistence of leptospiral DNA in serum 12 days after the onset of illness in patients with systemic symptoms, but some patients (28.5%) maintained it to the week 3 of the disease and one culture-confirmed case reached the maximum of 56 days. Bal et al. also reported the persistence and the shedding of leptospires in late samples of urine by using a PCR assay (2). As the persistence of leptospires in biological fluids was observed after treatment with β-lactamines, we can consider the hypothesis of an intracellular location of virulent leptospires. This illustrates the invasive abilities of virulent leptospires which are also able to remain located in areas protected from the immune response, such as meninges (32) or eyes (1, 29). Immunity to leptospirosis is mediated mainly by humoral mechanisms (14), and the presence of virulent *L. interrogans* in renal cells, where the leptospires would be protected from specific and nonspecific defense mechanisms, could explain this persistence in the host animal. This bacterial persistence is also well established for other spirochetes, such as *Treponema pallidum* in syphilis and *Borrelia* spp. in Lyme disease (4).

Entry of a pathogen into a host cell is one of the essential elements of microbial pathogenicity. We investigated the mechanism of entry of virulent leptospires into J774A.1 and Vero cells, using various specific inhibitors. The microfilament system is not required for internalization of virulent leptospires, since our results allow us to conclude that cytochalasin D does not significantly prevent leptospire entry. Nocadazole partially inhibited entry of virulent leptospires. Receptor-mediated endocytosis is a pathway by which extracellular molecules (15), viruses (18), and bacteria (9, 16) are internalized. After attachment at specialized regions of the plasma membrane (coated pits), the particles are progressively engulfed by the cell (34). Virulent leptospires are endocytosed via coated pits, and a transglutaminase inhibitor, such as monodansylcadaverine, inhibits their entry into host cells. This result is to be compared with that for another spirochete, *Borrelia burgdorferi*, which attached to coated pits on the plasma membrane of Vero cells in an electron microscopy study (16). Our results indicate that internalization of virulent leptospires appears to take place by a mechanism that is more dependent on the functions of receptor-mediated endocytosis via coated pits and less dependent on the functions of microtubules. The nocada-

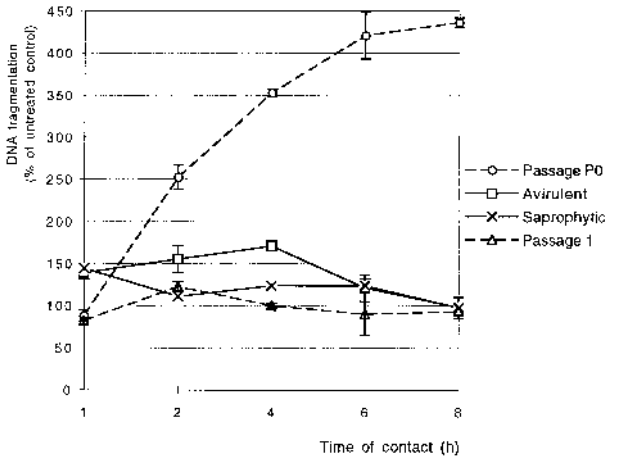

FIG. 5. DNA fragmentation in J774A.1 cells infected with virulent *L. interrogans* serovar icterohaemorrhagiae (P0 and P1), its avirulent variant, and saprophytic strain Patoc I (*L. biflexa* serovar patoc). DNA fragmentation was monitored by a quantitative sandwich enzyme immunoassay as described in the Materials and Methods. Results are the means of duplicate experiments ± standard errors (bars).

zole effect is likely to be indirect. Indeed, since microtubules are involved in the vesicular transport, we might speculate that nocadazole partially prevents the internalization of virulent leptospires by interfering with the modeling of coated pits. However additional factors, perhaps bacterium-derived, could be required, since complete inhibition was not observed. Electron microscopy studies would have to be performed to correlate with our findings with inhibitors. Other workers have reported the presence of intracellular virulent leptospires in thin electron microscopy sections 2 to 3 h after infection of mouse fibroblast (L929) monolayers (43). Those authors suggested that leptospires can enter the cells through damaged cell membranes. Conversely, in our experimental model eukaryotic cells are undamaged according to the trypan blue exclusion test.

Apoptosis or programmed cell death is a mechanism of controlled cell deletion and represents the end point of a genetically determined program (24, 25). PCD occurs in physiological processes but also in various pathological conditions such as cancer, immune system dysfunction, or infectious disease (5) but has never been documented for leptospires. Apoptosis plays an important role in the maturation of the immune system (5), and some pathogens are known to induce PCD in cells of the immune system (26, 47), altering immunological functions. Leptospires spread rapidly from the site of entry, via lymphatics to the bloodstream, where they circulate to all tissues. Avirulent leptospires which reach the bloodstream are cleared rapidly by reticuloendothelial phagocytosis, but virulent organisms survive by evading phagocytosis (14). Our data suggest that after entry into the organism, macrophages could undergo apoptosis as an in vivo mechanism to escape to the nonspecific activity of phagocytic cells before reaching target organs and persisting in host cells. Differences in induction of apoptosis between virulent and avirulent or saprophytic strains may be correlated to differences in their ability to gain access to the intracellular compartment. Indeed, located in the cytosol, virulent leptospires can easily enhance DNA fragmentation. Apoptosis of J774A.1 cells was also induced in our laboratory by a primary culture isolated from a human (data not shown). Apoptosis of Vero cells was not evidenced in this study; however, previous investigations allow us to suspect this phenomenon in other epithelial cells. Using electron microscopy, Miller and Wilson (31) studied the relationship of pathogenic leptospires with hepatic parenchymal cells (HPC) in the livers of hamsters previously infected and observed morphological changes typical of cells undergoing apoptosis cell death: a marked decrease in the volume of the cytoplasm, the appearance of rounded cells, the shrinkage of mitochondria, the frayed but intact cytoplasmic membrane, and the integrity of the cells. Moreover, the disarrangement of HPC was not accompanied by inflammatory cells, and those morphological changes were contemporary with the observation of intact leptospires in the cytoplasm of HPC. Further studies to investigate the in vivo apoptosis in other target organs or tissues involved in leptospirosis, such as the liver or blood vessels, will be performed.

The gradual loss of virulence of pathogenic leptospires after several passages in vitro is a classical datum which has been evidenced in animal models (14). To our knowledge, this phenomenon was never considered in previous in vitro studies of the interactions between pathogenic *Leptospira* and eukaryotic cells. Our data demonstrate that subculturing quickly impairs the virulence of isolates with a significant decrease, in six in vitro passages, of the invasion of Vero cells and of the active penetration in macrophages. In addition, the evidence of the apoptotic effect on J774A.1 is limited to the primary isolation of leptospires from infected guinea pigs.

In conclusion, we demonstrate that J774A.1 and Vero cells provide a useful in vitro model to study interactions of leptospires with phagocytic and epithelial cells. The invasion of Vero cells and the active early penetration and induction of apoptosis of macrophages are correlated with the virulence of *Leptospira*. Our data point out the necessity to use primary isolates to study the pathogenesis of leptospirosis. Further investigations should concern the identification of both *L. interrogans* and target host cell surface molecules implicated in mediating adherence, the determination of the cellular structures implicated in the internalization of virulent leptospires, and the possibility of an intracellular replication of these bacteria.

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