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Detection of newly emerging human ehrlichioses would benefit greatly from the commercial availability of a simple, standardized, and economical confirming diagnostic test. A serological dot ELISA dipstick (DS) test meeting these criteria, produced by Integrated Diagnostics, detects anti-rickettsial antibodies with sensitivity and specificity comparable to IFA. We explored the feasibility of producing similar DS for recognizing antibodies to pathogenic ehrlichiae by developing methods for the bulk cultivation and purification of 2 ehrlichia species (E. sennetsu, E. chaffeensis) and by producing and testing prototype DS. Large quantities of E. chaffeensis and E. sennetsu were successfully grown in Vero cells; inhibition of host cell division by daunomycin did not affect ehrlichial replication and permitted harvest from cultures of 100%, very heavily infected cells. Renografin gradient purification of E. chaffeensis was refined to optimize DS antigen yields; preliminary results suggest that antigen yields may be further increased by treating infected cells with Triton X-100. DS made with purified E. chaffeensis and HGE (human granulocytic ehrlichiae) antigens evidenced some cross reactivity when tested with anti-E. chaffeensis and -HGE convalescent sera, but reactions with control sera (from normal and rickettsia-, flavivirus-, and auto-immune donors) were largely negative. Thus we have demonstrated the technical feasibility of producing ehrlichial DS suitable for extensive field testing for sensitivity and specificity. These should provide a rapid, field-deployable, and easily performed and read alternative to IFA.

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Development of a dot ELISA for the detection of human antibodies to ehrlichiae

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INTRODUCTION

The family *Rickettsiaceae* includes several arthropod-borne, obligately intracellular bacteria (including the genera *Rickettsia, Orientia, Coxiella,* and *Ehrlichia*) which are significant human pathogens with wide geographic distribution. They cause acute febrile diseases which often are self-limiting but which can have long-term sequelae or be fatal if not treated. The true incidence of rickettsial and ehrlichial diseases is vastly underrated because they often are not diagnosed or are misdiagnosed (27; 44). Most of these diseases are zoonoses, with animals serving as hosts for the arthropod vectors, thereby amplifying rickettsial or ehrlichial growth and enhancing their distribution. Diseases caused by the *Rickettsiaceae* have a wide impact in many regions of the world; some have been recognized for hundreds of years while the significance of others, such as the ehrlichiae, is just emerging.

Identifying outbreaks of rickettsial and ehrlichial diseases is important to both their prevention and treatment. Unlike free-living bacteria, however, isolation of these infectious agents from human patients is expensive, time-consuming, and requires special facilities for handling hazardous organisms. Identification by PCR of rickettsiae and ehrlichiae in tissues requires expertise beyond the scope of clinical diagnostic laboratories. For these reasons, serological diagnosis, although generally retrospective, is usually preferred, and the serological method of choice is often the indirect fluorescent antibody (IFA) assay. Although the IFA test is considered sensitive and accurate, the source of antigen slides can be problematic, and reading the results requires a fluorescence microscope and considerable expertise.

To provide a serological test system suitable for easily trained individuals with no access to special equipment, Integrated Diagnostics (INDX®) has developed a commercial dot enzyme-linked immunosorbent assay (dot ELISA) for the detection of antibodies to *R. typhi, R. rickettsii, R. conorii, O. tsutsugamushi,* and *C. burnetii,* among other infectious agents (37, 68, 49, 51, 60, 64, 65, 75). The original DIP-S-TICKS® format incorporates serial dilutions of purified rickettsial antigen spotted on nitrocellulose dipsticks (DS) for testing a single dilution of serum. The DS are passed through a series of 4 reaction cuvettes containing serum diluent, enhancing solution, enzyme-conjugated anti-human IgG+IgM antibody, and a chromogen-

ic enzyme substrate. Positive results are read as discrete purplish dots. Although only a single serum dilution is tested, the antigen concentration detected by the test serum is proportional to the antibody titer (37, 65, 75) and thus the DS provide a semi-quantitative assay. The DS tests' advantages lie in their relative rapidity, good sensitivity and specificity, and the ease with which they can be read. No special equipment is needed, but the assay must be done at 50°C for optimum turn-around time and sensitivity. The DS test is especially well suited to unsophisticated settings such as often occur in developing countries or in military field deployments.

The recognition in the 1990's of at least 2 new ehrlichia species pathogenic to humans (1, 3, 11, 18, 24, 50) suggested a need for expanding the DS specificity to include these infectious agents. To explore the feasibility of doing so, it was necessary first to scale up production and purification of ehrlichiae and then to test the purified antigens on prototype DS. Methods for ehrlichia culture, purification, and diagnosis are still being developed because appreciation of their role in human illness is so recent. While ehrlichiae were first identified as veterinary pathogens, 3 species are now recognized as causing human disease: *E. chaffeensis*, the agent of human granulocytic ehrlichiosis (HGE), and *E. sennetsu*; and isolation of an *E. canis*-related strain (VHE) from a Venezuelan patient has just been reported (50) (TABLE 1).

Human ehrlichiosis in the U.S. was first suggested by IFA reactions of human sera with the canine pathogen *E. canis*. In 1991, an antigenically related but distinct human isolate was cultured from a mildly ill individual and subsequently designated *E. chaffeensis* (Arkansas strain) (1, 18). Other strains have since been isolated (13, 26). *E. chaffeensis* is monocytotropic and is in the *E. canis* genogroup. HGE was first isolated from a human patient in the upper Midwestern U. S. in 1994 (11). It is closely related if not identical to the veterinary pathogens *E. phagocytophila* and *E. equi*, and it is the first granulocytotropic ehrlichia shown to cause disease in humans. *E. sennetsu*, identified as a human pathogen in the 1950's, causes a relatively mild infection so far as known, with symptoms similar to those of infectious mononucleosis. Its known geographic range is restricted to Japan and perhaps parts of Southeast Asia (55); however, it has not been extensively looked for. *E. sennetsu* is related to *E. risticii*, the agent of Potomac horse fever.

Similar to other *Rickettsiaceae*, ehrlichiae are small, double-membraned coccobacilli. Unlike *Rickettsia* and *Orientia* species, which grow free in the host cell cytoplasm or nucleoplasm, however, ehrlichiae multiply within membrane enclosed phagocytic vacuoles (2, 43). After individual ehrlichiae enter a cell, they multiply within the phagosomal vacuole to form membrane-enclosed, densely packed groups termed morulae. The morulae increase in size over time as the ehrlichiae replicate and they eventually release their contents of individual organisms which may then infect other cells. In infected tissue cultures, ehrlichiae apparently are released from intact cells because the percent of cells infected may gradually increase before any loss

of viable cells is noted; infected cells may contain hundreds of organisms before bursting. Thus 2 types of ehrlichial inclusions are characteristically seen in infected cell cultures: individual bacteria and morulae. Ehrlichiosis may be diagnosed in peripheral blood smears of infected patients by finding characteristic morulae in monocytes (*E. chaffeensis*, *E. sennetsu*) or granulocytes (HGE). However, infected circulating cells may be rare, so the absence of morula-containing cells cannot be used to rule out infection.

The basis for the in vivo cell tropism of the granulocytic and monocytic ehrlichiae is unknown. In tissue culture, however, the monocytotropic ehrlichiae can be propagated in a variety of primary and continuous cell lines, including fibroblast as well as monocyte-derived. HGE, on the other hand, has only recently been cultured in vitro (15, 23, 25, 28, 46, 54), mostly in the promyelocytic leukemia HL-60 cell line. Methods for growing ehrlichiae in cultured cells are similar to those long employed for rickettsia production. Quantitative aspects of the ehrlichia growth cycle have not been studied extensively, in part due to the impossibility of counting the ehrlichiae within morulae and the tedium involved. Nonetheless, from data based on rough estimations of the number of *E. chaffeensis* per morula (5), one can approximate a doubling time of 12 hr, which is in the range of rickettsial generation times (30, 45, 71, 72). Infectious plaque formation in various cell lines has been reported (9, 14) but not used much as a quantitative assay.

Ehrlichiae have been purified by centrifugation through Renografin (12, 67, 68) or Percoll (69) density gradients, by centrifugation through Renografin (26) or sucrose (10) layers, or by passage through Sephacryl S-1000 columns (53). Ehrlichiae collected from Renografin gradients are still contaminated by host cell debris, however, and many are still contained within host membrane-bound vacuoles (67, 68). Presumably, the same is also true for ehrlichiae purified by the other methods. While organisms purified by the above methods are suitable as antigen for immunoblotting (10, 26, 53, 58), which is done after solubilization in SDS, it seems likely that antigen preparations not thus treated with detergent would have reduced yields because ehrlichiae within vacuoles should not be accessible to antibody.

Development of a commercial serological assay for anti-ehrlichia antibodies requires first an economical means of producing sizable quantities of purified antigen. Here we report our progress in developing methodology for the bulk culture and purification of monocytotropic ehrlichiae and the results of tests performed to evaluate ehrlichia antigen suitability for a DS assay using human sera. We found the anti-mitotic drug daunomycin (20, 30) to be useful for inhibiting Vero cell multiplication without affecting *E. chaffeensis* replication and determined that certain other additions to the culture medium did not adversely affect the ehrlichia yield. The standard procedure for Renografin density gradient purification was also examined in order to optimize antigen yield. Finally, purified antigen applied to DS was tested

and found capable of identifying anti-ehrlichia antibodies in human convalescent sera. The results indicate the feasibility of producing ehrlichia-specific DS satisfactory for extensive field testing of sensitivity and specificity. These should provide a rapid, field-deployable, and more easily performed and read alternative to IFA.

MATERIALS AND METHODS

Cell culture. Vero cells (line CCL81; African green monkey hypodiploid fibroblasts) were obtained from the American Type Culture Collection (ATCC; Rockville, Md.) and maintained in RPMI 1640 medium supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 5% fetal bovine serum (FBS), and 2 mM Glutamax I (L-alanyl-L-glutamine, a stable glutamine substitute), all purchased from Life Technologies, Inc., Grand Island, N.Y. (TCM). Other continuous cell lines (also purchased from ATCC) were HEL 299 (CCL 137; human embryonic lung diploid fibroblasts) and HL-60 (CCL 240; human promyelocytic leukemia cells). These cells were cultured in TCM containing 10% FBS instead of 5%; the medium for HEL 299 cells also contained 1 mM sodium pyruvate (Life Technologies). All cultures were incubated at 35 to 37°C in a humidified atmosphere of 5% CO_2 in air.

Ehrlichiae. *Ehrlichia sennetsu* (Miyayama strain), originally isolated from human blood in 1953, was purchased from ATCC (VR-367) as a frozen suspension of infected BS-C-1 cells. *Ehrlichia chaffeensis* (91HE17 strain), recovered from a severely ill patient, was kindly provided by J. S. Dumler of Johns Hopkins University Medical Institutions (26). This isolate is very similar to the Arkansas strain both genetically (99.9% 16S rRNA sequence identity with Arkansas strain 16S rRNA [26]) and antigenically (although a difference in a nonimmunodominant protein has been detected with a monoclonal antibody [25]). HGE was obtained from 2 sources: (i) infected horse blood (46) from J. E. Madigan and J. E. Barlough of the University of California at Davis School of Veterinary Medicine; and (ii) HL-60-grown and purified ehrlichiae (human isolate 96HE27) from J. S. Dumler (through a materials transfer agreement).

Ehrlichia cultivation. *E. chaffeensis* and *E. sennetsu* were passaged in daunomycin-inhibited Vero cell cultures in 225 cm^2 (T225) culture flasks following the general method described for passage of rickettsiae (30, 72). Culture flasks containing heavily infected cells were shaken with glass beads to release the cells into the TCM, and the cells and TCM were then centrifuged at 12,000 x g for 25 min to pellet the cells and any free ehrlichiae. The pellets were resuspended in a small volume of TCM and sonicated briefly (Model 250 sonifier with cuphorn attachment, Branson Ultrasonics Corp., Danbury, Conn.) to disrupt the cells. This mixture was added to suspended, uninfected Vero cells (final concentration of 3×10^7 uninfected cells per ml) and incubated for 1 hr at 35°C with frequent gentle shaking to keep the cells in

suspension. The ratio of infected to uninfected cells was 1 to 3-10. Following the 1 hr incubation, the cells were diluted in TCM containing 0.4 μg daunomycin (Sigma Chemical Co., St. Louis, Mo.) per ml (TCM-D) and seeded into T225 flasks (3×10^7 cells per flask) or tissue culture slides (5×10^4 cells per ml, 0.3 ml per well of an 8-chamber tissue culture slide ([Nunc, Inc., Naperville, Ill.]). The cultures were fed with fresh TCM-D every 3-6 days.

The infection was monitored by (i) periodic removal of culture slides for fixation in methanol and staining with Giemsa or acridine orange and (ii) observation of the flask cultures for cytopathic effect (CPE). The infected cells were passaged when CPE was rated 1+ to 3 + on a scale of 1 to 4+, corresponding to detachment of 50-75% of the cells, or when the fixed and stained cells were seen to be very heavily infected. Optimally, this occurred a day before complete lysis of the cultures, when nearly 100% of the cells were very heavily infected.

When ehrlichiae were to be frozen instead of passaged directly in fresh Vero cells, the 12,000 x g pellet (above) was suspended in modified attachment-penetration medium (BAPG; 10 mM potassium phosphate buffer [pH 7.3], 2 mM L-glutamine or Glutamax I, 45 mM NaCl, 62.5 mM KCl, 10 mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.3% [wt/vol] bovine serum albumin [BSA], 0.1% [wt/vol] glucose) (30) at a concentration of 1 T225 flask's contents per ml and frozen slowly to -100°C.

Ehrlichia and host cell growth studies. To assess the number of Vero cells in the Giemsa-stained slide cultures, cells in 4 sets of 25 to 50 randomly chosen, 1000x microscope fields in duplicate cultures were counted and recorded as mean number of cells per field (30). For ehrlichia quantitation, a minimum of 3 replicates of 100 cells was counted for each time point and condition. To quantitate ehrlichia growth, culture slides were examined for the presence of morulae or ehrlichiae (number of morulae plus approximate number of individual organisms); the results were expressed as (i) percent cells containing morulae or ehrlichiae, (ii) mean number of morulae or ehrlichiae per cell, (iii) mean number of morulae or ehrlichiae per infected cell, or (iv) mean number of morulae or ehrlichiae per 1000x microscope field (30, 71, 72). Morulae rather than individual ehrlichiae were often quantitated due to the inherent difficulty of identifying and counting the latter, but it should be noted that (i) over time the morulae increase in size as well as in number and (ii) this increase is not accounted for in the overall enumeration. Moreover, just counting morulae does not account for the accumulation of individual organisms.

Ehrlichia purification. The method for purifying ehrlichiae by Renografin density gradient centrifugation was based on that used for rickettsiae (31, 33, 45, 66). Infected Vero cell cultures frozen in BAPG were rapidly thawed, the cells were disrupted by brief sonication, and the ehrlichiae were partially purified by differential centrifugation. For this purpose, the disrupted cells were centrifuged at 200 x g for 5 min and the supernatant was transferred to a fresh tube. The 200 x g pellet was sus-

pended in BAPG, resonicated and centrifuged again at 200 x g for 5 min. The second 200 x g supernatant was added to the first and the 200 x g pellet was subjected to a third round of sonication and centrifugation. The pooled supernatants were centrifuged at 12,000 x g for 25 minutes (JA-14 or JA-20 rotor in a Beckman JT-21 centrifuge) and resuspended in BAPG; the suspension was kept relatively dilute to foster separation of ehrlichiae and host cell components (5 ml for each equivalent of 10 T225 flasks of infected cells). The partially purified ehrlichiae were incubated with 10 μg DNase I (15,000 units per mg protein; Sigma) per ml (58, 68, 69). Each 5 ml of suspension was layered over a 3-step 25-ml Renografin gradient made with 19%, 30%, and 45% Renografin (E. R. Squibb and Sons, Inc., Princeton, N. J.) in BAPG from which the BSA, glucose and divalent cations had been omitted (APG^-). Centrifugation was at 27,000 x g for 1 hr (SW28 rotor in a Beckman L5-75 ultracentrifuge). Visible bands were collected at the 19%-30% Renografin interface (equivalent to rickettsial T bands, density 1.15 g per cc [25% Renografin]) and at the 30-45% interface (equivalent to rickettsial L and H bands, densities 1.20 and 1.23 g per cc, respectively [35% and 39% Renografin]) (31, 33, 66). The bands were pooled, washed by centrifugation in APG (BAPG without the BSA), suspended finally in APG, and frozen at -100°C.

IFA. To make antigen slides, ehrlichia-infected Vero cells were removed from culture flasks with trypsin-EDTA (Life Technologies), washed, and suspended in TCM at 5 - 7.5 x 10^5 cells per ml. The infected cells were dispensed with a repeating micropipette in 25 μl drops onto teflon-coated glass slides containing 12 6-mm wells. The slides were then incubated in a well-humidified CO_2 incubator for 3 hr during which time the cells attached and spread on the glass. Slides were then rinsed in two changes of physiologic saline, partially dehydrated in acetone and then fixed in a second acetone jar for 10 min at room temperature. After air drying, the slides were stored in sealed plastic slide boxes containing silica gel dessicant at -100°C.

The slides were tested as instructed by the INDX IFA slide package insert: They were incubated with sera diluted in PBS for 30 min at room temperature, washed in PBS, and then exposed to fluorescein isothiocyanate-conjugated anti-human IgM + IgG antibody for another 30 min at room temperature. After rinsing, the slides were treated with eriochrome black (INDX) for 2 min, rinsed, and read with a fluorescence microscope.

Dot immunoassay. Prototype dot ELISA antigen strips were made according to standard methods employed at Integrated Diagnostics for the production of DIP-S-TICKS. The DS format is a nitrocellulose strip with 6 wells to which 1.5 μl of selected antigen dilutions in phosphate buffered saline (PBS) are applied. For development purposes, serial 2-fold dilutions of antigen were applied, and the antigen-dotted DS were air-dried, blocked with 13.5% (wt/vol) nonfat dry milk in deionized water, rinsed in deionized water, and air-dried again.

Reagents for testing the DS were supplied by Integrated Diagnostics and the methodology followed directions packaged with the DIP-S-TICK test kits. Each antigen-coated DS was tested by processing through a series of 4 reaction cuvettes containing 2 ml of (1) test serum diluent plus 10 µl test serum (to give a 1:200 dilution), (2) "enhancer" solution to enhance removal of extraneous proteins; (3) conjugate (alkaline phosphatase conjugated goat anti-human IgG [H + L chains] and IgM [µ chain], and (4) developer (alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitro blue tetrazolium chloride). Incubation at 50°C in each of the 4 cuvettes was for 5, 5, 15, and 5 minutes respectively, with rinsing in deionized water between each reaction step. After the final rinse and the DS were dried, positive reactions could be seen as purple dots with distinct borders.

Human sera. Immune sera were obtained from confirmed cases of monocytic and granulocytic ehrlichiosis and donated by Drs. E. Hilton and J. Devoti (Long Island Jewish Medical Center, New Hyde Park, N. Y.) and J. S. Dumler. Ehrlichia antibody-negative sera were from a panel provided by Integrated Diagnostics; these included sera with antibodies to several other infections (rickettsial, bacterial, viral) and to autoantigens as well as sera from apparently healthy donors.

RESULTS: PROPAGATION OF EHRLICHIAE

Effect of daunomycin on *E. chaffeensis* replication in Vero cells. Inhibition of host cell replication increases the ratio of intracellular bacteria to cells and may lead to an overall increase in yield of microorganisms. As an alternative to host cell irradiation, the eukaryotic inhibitor daunomycin has been used to enhance the production of *O. tsutsugamushi* in cultured cells (30). Although daunomycin does not inhibit rickettsial replication (30), it was important to determine if ehrlichial growth was inhibited or suboptimal in it. As a preliminary step, the lowest inhibitory dose of daunomycin in uninfected Vero cells was confirmed by suspending the cells in TCM with different daunomycin concentrations and following their proliferation in culture slides. It was found that daunomycin concentrations of 0.25 µg per ml or higher were completely inhibitory under the conditions tested; 0.60 µg per ml appeared to be toxic by day 5 and 0.15 µg per ml inhibited Vero cell replication substantially but not completely (FIGURE 1). As previously described (30), the daunomycin-treated Vero cells enlarged over time without accumulating the multiple nuclei which often result from inhibition by irradiation or colchicine and remained healthy looking.

Next, the effect of daunomycin on ehrlichial replication was examined. Vero cells were infected in suspension as usual and aliquots were diluted in TCM with or without antibiotic to give final concentrations of either 0.40, 0.25, 0.10, or no µg daunomycin per ml. Slide cultures were fixed and stained at intervals for assessment of Vero cell and ehrlichia replication. Because 2 daunomycin concentrations (0.40 and

0.25 μg per ml) completely inhibited Vero cell replication and had the same effect on the examined parameters of ehrlichia growth, the results from these 2 concentrations were combined in the final figures. Also for the sake of simplicity, the data from treatment with 0.10 μg daunomycin per ml were omitted from the figures; its effect was generally intermediate between the effects of the higher concentrations and the untreated controls.

As expected, the Vero cell numbers in the daunomycin-treated cultures remained relatively constant throughout the experiment (FIGURE 2A) while in the absence of the drug the cells increased 7-fold during the 6 days studied. The data also show that over this period, infection of Vero cells with *E. chaffeensis* had no discernible effect on their response to daunomycin nor on their growth in the absence of daunomycin (compare FIGURES 1 and 2A).

E. chaffeensis replication in Vero cells followed the same general pattern reported by others. The percent cells infected increased without a discernible lag period, and the ehrlichiae tended to spread just to neighboring cells, forming foci of infection. As they multiplied within vacuoles, the apparently individual organisms in early cultures were replaced by morulae of increasing size. By the fifth day post infection the percent cells containing morulae had decreased somewhat (FIGURE 2B); this coincided with an increase in the percent cells just containing particles which appeared to be individual ehrlichiae, possibly reflecting maturation and bursting of morulae and reinfection with individual organisms (FIGURE 2C).

The effect of daunomycin on ehrlichia replication was assessed in several ways: (i) The percent cells containing morulae was higher in the daunomycin-treated cultures after the first day post-infection (FIGURE 2B) as was the total percent cells infected (not shown). This might be expected as uninfected cells are continuously added to the untreated culture due to replication. (ii) The mean number of morulae per cell was higher in daunomycin-treated cells (FIGURE 2D). (Again, note that the untreated cultures contained more cells.) (iii) The average number of morulae per infected cell was similar under both conditions, however (except on day 6 when it was slightly higher in the treated cultures). This indicates that in any given cell *E. chaffeensis* replication was not substantially affected by daunomycin treatment (FIGURE 2E). (iv) When the total ehrlichia yield per culture was calculated by multiplying the number of morulae per cell by the number of cells per microscope field, daunomycin was seen to have no effect (FIGURE 2F). Thus the daunomycin treatment did not alter the replication or yield of ehrlichiae but it did increase significantly the ratio of ehrlichiae to cells. This should significantly aid in the purification of the organisms. The morula doubling time in this experiment was calculated by regression analysis to be 24 hours.

These results suggested the potential utility of inhibiting ehrlichia-infected HEL 299 and HL-60 cell replication as well, since we were interested in trying to cultivate

HGE in these lines. To determine the optimum doses for these cell types, both were treated with various concentrations of daunomycin and their replication was monitored either by counting cells per field (HEL 299 monolayers in chamber slides) or cells per ml (HL-60 suspension cultures). The minimum dose required for complete inhibition of HEL 299 cultures was 0.02 μg per ml (FIGURE 3), about a tenth that for Vero cells and the same as that previously found for $P388D_1$ cells (30). However, even this low dose was toxic for HL-60 cells; in fact the inhibitory but nontoxic concentration range was so narrow (and the cell growth rate so slow) that the use of daunomycin in these cultures seems unwarranted.

Effect of other TCM components on *E. chaffeensis* replication in Vero cells. The dipeptide L-alanyl-L-glutamine (Glutamax I) has been used successfully as a more stable form of glutamine for culturing a variety of cells (Life Technologies catalog) including rickettsia-infected Vero cells (32). Particularly because glutamine is a major energy source for ehrlichiae (67, 68, 69), it was important to see if Glutamax in TCM also supported ehrlichial replication as well as L-glutamine in daunomycin-inhibited Vero cells. For this purpose, Vero cells infected in suspension were centrifuged and suspended either in TCM-D or TCM-D containing 2 mM glutamine instead of 2 mM Glutamax before distribution to tissue culture slides (FIGURE 4). In both media, the percent cells infected (morulae plus individual organisms) increased steadily from the first day after infection (FIGURE 4A). Through day 6 ehrlichia replication in glutamine or Glutamax was similar; however, by day 7 the infection was slightly heavier in cells cultured in Glutamax. The slight preferability of Glutamax to glutamine late in infection was also found in a repeat experiment and may reflect the greater stability of Glutamax. Glutamax seemed to enhance Vero cell survival relative to glutamine (FIGURE 4B). Thus when the number of infected cells per culture was calculated (fraction of cells infected times number of cells) the ehrlichia yield in Glutamax was slightly higher throughout the course of infection (FIGURE 4C). We therefore concluded that substitution of Glutamax for glutamine was acceptable (and probably preferable).

In similar experiments, the presence of HEPES buffer did not affect the replication of ehrlichiae (FIGURE 5), nor did it influence the number of cells in the cultures (not shown).

Serial passage of *E. chaffeensis* in Vero cells. Some parameters of the serial passage of *E. chaffeensis* in daunomycin-inhibited Vero cells were examined in an attempt to optimize the procedure and predict its outcome. Split ratios (number of new flasks made from each infected flask) ranged from 1 to 10, and infected flasks were harvested when the CPE (expected to be a rough measure of the level of infection) was between + 1 and + 3 and/or when nearly 100% of the cells were seen to be heavily infected. The day of harvest (passage duration) varied from 2 to 11 days. Analyzed over the entire observation period (28 serial passages), neither the degree of

CPE of the inoculum culture nor the split ratio could be used to definitively predict the subsequent passage day (FIGURE 6A). However, when cultures with split ratios of 2-4 were analyzed, the time between passages did decrease as the passage number increased (FIGURE 6B), suggesting a possible adaptation of *E. chaffeensis* to replication in Vero cells.

Cultivation of *E. sennetsu* in Vero cells. *E. sennetsu* was well suited for propagation in daunomycin-inhibited Vero cells. One quarter ml of the *E. sennetsu* from ATCC was mixed with 1×10^7 Vero cells and put into culture as described in Materials and Methods. About 1% of the cells were initially infected and by day 20 about half the cells were infected. Microscopy revealed numerous organisms which were loosely distributed throughout the cytoplasm and which occurred singly and in small clumps (morulae). The *E. sennetsu* morulae never became as large and distinct as the *E. chaffeensis* morulae. Like *E. chaffeensis*, *E. sennetsu* was observed in the slide cultures as foci of infection, indicating that these ehrlichiae also spread most readily to neighboring cells. The cells became very heavily infected before rounding up and eventually detaching from the culture flask. Disruption of heavily infected cells could be observed microscopically. Through 11 subsequent serial passages, the mean passage day was 7.0 ± 2.0 (S. D.); there was no evidence of its decreasing with passage number.

Attempts to propagate HGE in cell culture. Following recent reports on the successful cultivation of HGE in cell culture (28, 46), we attempted to isolate it in HL-60, HEL 299, and Vero cells from HGE-infected horse blood. Forty to 50% of the polymorphonuclear leukocytes in the inoculum contained 1 or 2 morulae. Both whole blood and buffy coat cells derived from it were inoculated in various volumes into HL-60 and Vero cell cultures, following the published procedures. In addition, on day 11, HGE-exposed HL-60 cells were added to HEL-299 monolayers to see if an infection could be transferred to the latter. About a week after exposure to leukocytes or HGE-exposed HL-60 cells, the monolayer Vero and HEL 299 cultures were treated with the appropriate daunomycin concentrations so that they could be maintained in healthy condition; the HL-60 cultures were not exposed to daunomycin. All cultures were fed regularly and counted and subcultured (HL-60 cells) or examined for any CPE (monolayer cultures). Tissue culture slide monolayer cultures or HL-60 smears were stained at intervals with Giemsa or acridine orange to look directly for organisms.

After 8 weeks in culture, ehrlichiae could not be detected in HL-60 cells by acridine orange staining or by immunofluorescent staining with anti-HGE human antisera. The HGE-exposed HL-60 cell cultures continued to replicate, although at a slightly lower rate than uninfected control HL-60 cells at first. Likewise, there was no evidence of HGE infection of either the Vero or HEL 299 cell cultures; both types remained healthy-looking monolayers and no morulae were observed after staining

with either Giemsa or acridine orange.

RESULTS: PURIFICATION OF EHRLICHIAE

Purification of *E. chaffeensis* in Renografin density gradients. Three-step Renografin density gradient centrifugation of partially purified *E. chaffeensis* resulted in 3 sharp ehrlichia-containing bands comparable in amount to those regularly obtained with rickettsiae. Thus visual inspection indicated that the ehrlichia yield was reasonable and the use of a 3-step gradient was satisfactory. However, microscopic examination of Giemsa-stained smears revealed the presence of amorphous material of undetermined (probably host) origin as well as of apparently intact, mostly individual, ehrlichiae in each fraction.

In an attempt to release more *E. chaffeensis* antigen from host cell material, we varied our methods of cell breakage before continuing with differential and then Renografin density gradient centrifugation. A crude way to evaluate the separation of rickettsiae and host components is to observe the relative amounts of material in each band, since the top T band contains a higher proportion of contaminating cell membranes than the L (light) or H (heavy) bands (31, 33, 66). Therefore, we compared the amounts of T and H+L bands during each ehrlichia gradient centrifugation. Disrupting similar pools of thawed, infected Vero cells by sonication or Dounce homogenization showed that the former procedure was more effective in terms of both (i) the proportion of H+L to T band (better purified to less purified) material and (ii) the yield of antigen as determined by DS titration of H+L band material. Thus, while the ratio of material in the H+L bands as compared to that in the T bands was 2-3 to 1 after sonic disruption, it was only about 1 to 1 after Dounce homogenization. Moreover, the relative yield of antigen (DS antigen titer times total volume) was about 3 times higher after sonication than after milder homogenization.

Preliminary Renografin gradient centrifugation of partially purified *E. chaffeensis* not treated with DNase resulted in the presence of abundant stringy material which interfered with collection of the ehrlichia bands. Digestion of the partially purified ehrlichiae with DNase I (15,000 units per mg protein) prevented this complication, as has been noted by others. Varying the DNase concentration between 10 and 200 μg per ml did not alter its beneficial effect; therefore, the lowest concentration was adopted in the final procedure. In addition, digesting the suspension just prior to loading it on gradients was as satisfactory as DNase treatment before the differential centrifugation step.

Because most investigators use a sucrose-based Renografin and ehrlichia diluent (sucrose-phosphate-glutamine; SPGn or SPK-GN) (26, 68, 69), we performed one purification comparing SPGn and the AP buffer system, which is the diluent used at INDX for purifying rickettsiae. The ehrlichiae banded similarly in both diluents and

the antigen yields in each gradient were identical.

Triton X-100 (TX), in the presence of $MgCl_2$, has been effective in separating *O. tsutsugamushi* from infected Vero cell material without disrupting all the rickettsial particles (31). To see if this procedure could be applied to ehrlichiae, a 200 x g pellet (intact cells, nuclei, cell debris) obtained from sonically disrupted *E. chaffeensis*-infected Vero cells was divided into 2 equal aliquots and treated with either 1% or 0.1%TX in HEPES-buffered 10 mM $MgCl_2$. The pellets obtained by subsequent centrifugation at 12,000 x g were washed, diluted in PBS, and titered on DS. Extraction in 1% TX yielded just a small amount of antigen, but the titer of particulate antigen extracted by 0.1% TX was substantial even though the starting material was a cell residue fraction from which a significant amount of ehrlichiae had already been removed. The potential for removing more antigen from infected cells by mild detergent treatment will be investigated further.

RESULTS: ANTIGEN ACTIVITY

Effect of sonication and detergent treatments of purified ehrlichiae on antigen activity. Because some ehrlichiae are still membrane-bound after the Renografin density gradient purification (67, 68), we attempted to disrupt any such vesicles either by extensive sonication of Renografin-purified ehrlichiae (H+L band) or detergent treatment to see if this would enhance their antigenicity. After sonication for 1, 2, or 4 minutes, the antigen was serially diluted, spotted on DS, and tested with antiserum. Sonication under these conditions did not increase antigen titers.

Two detergents were used in an attempt to expose more ehrlichial antigenic determinants: (i) **SDS** was tried with the knowledge that many ehrlichial antigens are stable in 2% SDS when tested by immunoblotting. (ii) **TX**, a much milder detergent, has been useful in extracting many (including rickettsial) antigens (21, 29, 34). Treatment was by two methods: Either (i) different amounts of detergent were incorporated into the antigen dilution buffer before application to DS or (ii) antigens were preexposed briefly to detergent and then diluted out in buffer only. None of these procedures significantly enhanced the antigen titers (FIGURE 7). Either type of treatment with SDS was inhibitory, the presence of SDS in the dilution buffer being more so. The inclusion of TX in the dilution buffer also inhibited antigen activity; this may have been due to interference with antigen binding to the nitrocellulose strips, since brief exposure to TX before dilution in PBS did not affect subsequent antigen activity.

RESULTS: DIPSTICK EVALUATION

Evaluation of antigen strength and suitability. *E. chaffeensis* and HGE DS made with serial antigen dilutions were tested with a panel of sera from ehrlichiosis-confirmed patients, presumed normal donors, and individuals with a variety of other

disease states. The results demonstrate the feasibility of using DS to detect ehrlichia-specific antibodies (TABLES 1 and 2). All 7 of the anti-*E. chaffeensis* sera reacted to one degree or another with the *E. chaffeensis* DS, while only 3 of the 5 anti-HGE sera did (TABLE 1). All of the anti-HGE sera reacted with the HGE DS, again to various extents, and 5 of the 7 anti-*E. chaffeensis* sera did also (TABLE 1). The cross reactivity between the 2 ehrlichial species conforms to that observed by us and others with IFA. None of the 4 control sera from presumed healthy donors gave positive reactions with either species of DS (TABLE 1). Most of the 13 disease-state sera tested were negative with both *E. chaffeensis* and HGE DS (TABLE 2); 3 (anti-*Rickettsia conorii*, anti-*Borrelia burgdorferi*, and anti-DNA) reacted with HGE DS. The extent to which these represent properties of the particular antiserum (e.g. reflecting a dual infection?) remains to be determined.

Preliminary evaluation of antigen specificity. To test ehrlichia DS stability, they are stored at ambient temperature and tested periodically. After 10 weeks there was no drop in *E. chaffeensis* antigen titer; long-term stability testing of these and HGE DS is ongoing.

DISCUSSION

Our ultimate goal is to produce and use purified antigen from *E. chaffeensis*, *E. sennetsu*, and HGE. The methodology borrowed from rickettsia procedures and refined in the present study seems seems generally applicable to all 3 species; despite our initial failure to cultivate HGE in HL-60, Vero, or HEL 299 cells, it has been grown this year by other investigators in HL-60 cells (15, 22, 23, 28, 54), and we will try again to do so in collaboration with Dr. Dumler. The HGE and *E. chaffeensis* antigens were purified by centrifugation through 30% Renografin (26) and 3-step Renografin density gradients, respectively. Similar purification of *E. sennetsu* on Renografin gradients should be straightforward, as it has already been carefully documented (67, 68). Thus we foresee no problem in producing satisfactory DS with the *E. sennetsu* we have already grown.

Our observations of monocytotropic ehrlichia replication in Vero cells conform generally to those made by others. *E. chaffeensis* and *E. sennetsu* have been cultured in a variety of cell lines, including, in the case of *E. chaffeensis*, Vero cell monolayers (9, 14). Therefore it was not surprising that *E. sennetsu* grew well in Vero cells too. We confirmed the gradual spread of ehrlichiae from cell to cell before any noticeable cell lysis and the formation of foci of infection in the cultures (9, 14). *E. chaffeensis* spread among daunomycin-inhibited Vero cells at about the same rate reported by Dumler et al. for the same isolate in HL-60 cells (26). Chen et al. (14) observed that the foci of *E. chaffeensis* infection of irradiated Vero cells contained a mixture of infected and uninfected cells and suggested that these cells are heterogeneous in their capacity to support *E. chaffeensis* growth. We, however, regularly

found 100% of Vero cells could be infected with either species when it was confirmed that no host cell proliferation occurred.

As is the case with rickettsiae (30, 32), the eukaryotic anti-mitotic drug daunomycin proved to be very useful in inhibiting host cell growth while not affecting *E. chaffeensis* replication. Holding the number of host cells constant increased the percent cells infected, thereby avoiding unnecessary dilution of ehrlichia antigen with host material. This effect has also been noted with *E. chaffeensis* in phorbol myristic acid-inhibited THP-1 cells (5), and others have irradiated cells before *E. chaffeensis* infection (14). Inhibiting host cells with daunomycin has advantages over irradiation in that it does not require expensive, hazardous equipment and doesn't cause the formation of multinucleated cells which may interfere with the microscopic recognition of intracellular bacteria (30). We have been able maintain healthy-looking Vero and HEL 299 cell monolayers for months in daunomycin-containing medium; this can be especially helpful when ehrlichia isolates are being adapted to tissue culture. Although the effect of daunomycin on proliferation of *E. sennetsu* in Vero cells was not systematically studied, these ehrlichiae grew at expected rates during treatment of infected cultures. The need to calibrate each cell line for effective daunomycin dose (30), confirmed in these studies, must be emphasized.

E. chaffeensis replication in cultured cells is susceptible to the buffering system and concentration of glutamine (26), which is a major ehrlichial energy source (67, 68, 69) as well as a vital nutrient for the cells. Successful substitution of Glutamax for glutamine provides a more stable medium component which may enhance long-term culture. Bolstering the sodium bicarbonate buffer system with HEPES had no effect on ehrlichia growth and no apparent effect on culture pH in the CO_2 incubator; its benefit lies in the extra buffering capacity outside the incubator, such as during the infection of suspended cells in a water bath. Neither of these changes to standard culture media have affected the replication of spotted fever, typhus, or scrub typhus rickettsiae either (30, 32).

Both *E. chaffeensis* and *E. sennetsu* can now be readily passaged in Vero cells. With the procedures employed, it is possible to infect 100% of the cells initially and to thereby synchronize the growth cycle; this maximizes the yield and shortens the passage duration. *E. chaffeensis* appeared to adapt to growth in Vero cells because the day of harvest (just before it was anticipated the cells would disrupt) decreased for the last several passages when the data for similar split ratios were examined. The *E. chaffeensis* growth studies described in Figures 2, 4, and 5 were done with ehrlichia Vero cell passage numbers 9 and 13; growth curves of early- and late-passage ehrlichiae have not been systematically compared to see if this is a true adaptation.

Purification of ehrlichiae by centrifugation through Renografin density gradients is less complete than that of similarly processed rickettsiae (16, 17, 66, 67, 68), but

no other method has been shown to be more effective. In the present study, Renografin purification provided satisfactory antigen for identifying ehrlichia-specific antibodies. Compared to rickettsial antigen production (32), however, the ehrlichial antigen yields from the gradients were disappointing. This was at least partly due to the retention of a lot of ehrlichiae in bands above the H+L bands and also in the 200 x g pellet fraction usually discarded in the pre-Renografin, partial purification stage. TX was used to try to separate ehrlichiae from Vero cell material. Under appropriate conditions, this detergent selectively permeabilizes cytoplasmic membranes, leaving gram-negative bacterial outer membranes and eukaryotic nuclear membranes intact (8, 34, 39, 56, 57). (Its effect on cytoplasmic membranes should be less important in the current context since immunodominant bacterial antigens are generally in the outer membrane.) In studies with *O. tsutsugamushi*, the *Orientia/Rickettsia* species most sensitive to detergent extraction (29, 31) and most difficult to purify, treatment of the organisms with 1% TX in 10 mM phosphate or HEPES buffer and 10 mM $MgCl_2$ was useful in removing extraneous material without an unacceptable loss of antigenic activity. As long as $MgCl_2$ was present, the major protein antigens remained primarily with a particulate rickettsial fraction recoverable either by high speed centrifugation or by sedimenting as H bands in Renografin gradients (29, 31). In contrast, Vero cell proteins were completely released by this treatment. In the present investigation, treating the 200 x g fraction obtained after sonic disruption of most of the infected cells with 0.1% TX in 10 mM $MgCl_2$ effected extraction of a considerable amount of additional antigen. This finding will be pursued as we continue to try to optimize antigen yields.

Previous studies with *O. tsutsugamushi* also showed that the BAP buffer system was superior to the common rickettsia sucrose-phosphate-glutamate (SPG; 7) diluent in separating rickettsiae from host cell components during Renografin density gradient purification; this was manifested by a greater proportion of rickettsiae sedimenting in H+L bands in BAP than in SPG (31). Such did not seem to be the case with ehrlichia purification however, suggesting differences in the surface properties of these organisms.

Selective extraction of host cell membranes in purified antigen preparations also could remove competing or masking components and thereby enhance ehrlichia antigenic reactivity. Attempts to accomplish this by mild detergent treatment of Renografin-purified antigen were unsuccessful. This failure may have been due at least in part to detergent interference with antigen binding on the DS. Likewise, more extensive sonication of the antigen also failed to increase the DS antigen titers. Retention of antigen activity after washing TX-extracted infected cells (above) still remains a promising approach to this question.

DS made with purified *E. chaffeensis* and HGE antigens reacted with anti-ehrlichia antisera and not with sera from apparently healthy control serum donors. Not

unexpectedly, some cross reactivity between the 2 species was noted. Its extent cannot be assessed without testing more sera, and this is planned in future studies which will include comparison of immunoblots as well. The IFA cross reactivity between *E. chaffeensis* and HGE in human convalescent sera sometimes noted is generally accompanied by lower titers to the heterologous species, and it is not strong enough to warrant use of one as a surrogate antigen for the other (4).

Three of the control sera (1 each of anti-*R. conorii*, -B. *burgdorferi*, and -DNA) also reacted with the HGE DS in our studies. The incidence of such dual reactivity with DS antigens remains to be determined by testing more sera. However, the lack of reactivity of the anti-*R. rickettsii* serum in our experiments supports other findings of no ubiquitous cross reactivity between ehrlichiae and spotted fever group or other rickettsiae (6, 23, 42, 59, 61, 76). Multispecific reactions of some ehrlichia-positive sera also may be due to dual infections (6, 19, 42, 47, 48, 62, 73). The potential for coinfection is supported by the fact that ehrlichiae share tick vectors with other significant human pathogens, including *Borrelia burgdorferi*, *R. rickettsii*, and *Babesia microti* (36, 41, 42, 63, 74).

It is possible to overcome some degree of cross reactivity by adjusting the DS antigen concentrations to exclude false positives. To address this, a larger number of antisera, ranging from weakly to strongly positive with homologous antigen, will be tested with homologous and heterologous ehrlichia DS, and the antigen concentrations which best distinguish the sera will be selected for the final DS composition. A further degree of specificity may be found in early sera by looking only at IgM antibodies (73). These approaches are planned for Phase II of our DS development.

CONCLUSIONS

Large quantities of *E. chaffeensis* and *E. sennetsu* were cultured in Vero cells by methods similar to those used at Integrated Diagnostics for rickettsia production. The methodology was validated by demonstrating that (i) ehrlichia replication was not inhibited by the anti-mitotic drug daunomycin, which is used to prevent host cell proliferation and thereby increase the yield of organisms per cell; (ii) substitution of Glutamax for the less stable glutamine in the culture medium did not adversely affect ehrlichia growth; and (iii) inclusion of HEPES in the culture medium as an additional buffering agent had no effect on ehrlichia propagation. These 2 species of rickettsiae have each been serially passaged for a number of times; adaptation of *E. chaffeensis* to Vero cells was suggested by our capacity to passage it more rapidly in later passage cycles, but this was not rigorously confirmed. The knowledge derived from this work permits production of ehrlichiae from 100%, heavily infected cultures in a predictable manner. During the course of this work the first successful in vitro cultivation of HGE was reported. Our attempt to isolate HGE from infected horse blood in cell cultures was unsuccessful; therefore, we ultimately used HL-60

cell-grown HGE provided by J. S. Dumler (22, 23, 28).

E. chaffeensis was purified by centrifugation through Renografin density gradients by methods adapted from those used at INDX for rickettsia purification. Antigen yields were maximized by determining the optimum conditions for host cell disruption and DNase digestion. Two buffer systems (one commonly used for ehrlichia purification and the other employed for rickettsia purification at INDX) were equally effective. Preliminary experiments suggest that treatment of infected cells with TX may release more ehrlichia antigen and thereby improve the yields. This possibility is being pursued. The HGE was purified by Dr. Dumler by centrifugation through a Renografin layer; there is no reason to anticipate any difficulties in purifying this or *E. sennetsu* by the methodology adapted in our laboratory for *E. chaffeensis* purification.

Antigen activity was tested after application of serial dilutions of purified *E. chaffeensis* and HGE onto DS. Based on our customary yields of rickettsia antigen, ehrlichia antigen titers were lower than anticipated; extensive sonication of the purified antigen or mild detergent (SDS, TX) treatments were ineffective in increasing the titers. Nonetheless, the *E. chaffeensis* and HGE antigen titer and purity were sufficient for producing DS of good quality. DS of both specificities were capable of identifying ehrlichia-specific antisera. Not unexpectedly, substantial cross reactivity between the 2 species was seen in tests with 7 anti-*E. chaffeensis* and 5 anti-HGE convalescent sera. Reactions with 17 control sera were minimal; all were negative except for 3 (1 anti-*R. conorii*, 1 anti-*B. burgdorferi*, and 1 anti-DNA) that bound to HGE (but not *E. chaffeensis*) antigen. The nature of the cross reactions will be addressed in the next phase of this study.

DS stability tests still underway indicate that the *E. chaffeensis* DS have retained full activity after storage at ambient temperature for 10 weeks. The stability assessments of DS of all 3 ehrlichia species will be continued for several months.

This work has demonstrated that ehrlichiae can be economically grown and purified in quantities sufficient for DS production. Studies showing the effectiveness of daunomycin and other culture medium components in supporting ehrlichia growth have been instrumental in this success, and refinement of standard purification methods has assured optimum recovery of ehrlichiae from Renografin gradients. Furthermore, a potential means of obtaining higher antigen yields has been identified; the usefulness of detergent extraction will be examined further.

The sensitivity and specificity of the ehrlichia antigen DS were sufficient to warrant further tests. The DS could distinguish ehrlichia-positive antisera from most other control sera tested. Cross reactions between the ehrlichia species need to be examined more fully with a much larger number of antisera; this is planned for Phase II of this study. In addition, the few cross reactions observed between ehrlichia and other infectious agents will be further explored to determine if they represent

dual exposures. Phase II will also include fine tuning of the antigen concentrations to reduce cross reactivity.

In summary, we have proven the feasibility of adding ehrlichia antigens to the DS we already manufacture to test for antibodies to rickettsiae and other infectious agents. The next phase of DS development will be to (i) adjust the DS antigen concentrations based on tests with a large number of retrospective sera solicited from clinical field sites, (ii) have the DS tested in regionally appropriate clinical field sites, and (iii) validate the DS tests by comparisons with IFA results and other methods.

REFERENCES

1. **Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson.** 1991. *Ehrlichia chaffeensis,* a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29**:2838-2842.
2. **Anderson, D. R., H. E. Hopps, M. F. Barile, and B. C. Bernheim.** 1965. Comparison of the ultrastructure of several rickettsiae, ornithosis virus, and *Mycoplasma a* in tissue culture. J. Bacteriol. **90**:1387-1404.
3. **Bakken, J. S., J. S. Dumler, S.-M. Chen, M. R. Eckman, L. L. Van Etta, and D. H. Walker.** 1994. Human granulocytic ehrlichiosis in the upper midwest United States. a new species emerging? J. Am. Med. Assn. **272**:212-218.
4. **Bakken J. .S, J. Krueth, C. Wilson-Nordskog, R. L. Tilden, K. Asanovich, and J. S. Dumler** . 1996. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. J. Am. Med. Assn. **275**:199-205.
5. **Barnewall, R. E., and Y. Rikihisa.** 1994. Abrogation of gamma interferon-induced inhibition of *Ehrlichia chaffeensis* infection in human monocytes with iron transferrin. Infect. Immun. **62**:4804-4810.
6. **Barton, L. L., J. E. Dawson, G. W. Letson, A. Luisiri, and A. J. Scalzo.** 1990. Simultaneous ehrlichiosis and Lyme disease. Pediatr. Infect. Dis. J. **9**:127-129.
7. **Bovarnick, M. R., J. C. Miller, and J. C. Snyder.** 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J. Bacteriol. **59**:639-341.
8. **Brawerman, G.** 1973. Isolation of RNA from mammalian cells, p. 1. *In,* D. M. Prescott (ed.), Vol. 7 Methods in Cell Biology, Academic Press, N. Y.
9. **Brouqui, P., M. L. Birg, and D. Raoult.** 1994. Cytopathic effect, plaque formation, and lysis of *Ehrlichia chaffeensis* grown on continuous cell lines. Infect. Immun. **62**:405-411.
10. **Brouqui, P., J. S. Dumler, D. Raoult, and D. H. Walker.** 1992. Antigenic characterization of ehrlichia: protein immunoblotting of *Ehrlichia canis, Ehrlichia sennetsu,* and *Ehrlichia risticii.* J. Clin. Microbiol. **30**:1062-1066.
11. **Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of

a granulotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32**:589-595.

12. **Chen, S.-M., J. S. Dumler, H.-M. Feng, and D. H. Walker.** 1994. Identification of the antigenic constituents of *Ehrlichia chaffeensis*. Am. J. Trop. Med. Hyg. **50**:52-58.
13. **Chen, S.-M., V. L. Popov, H.-M. Feng, and D. H. Walker.** 1996. Isolation and analysis of a new strain of *Ehrlichia chaffeensis*. Abstr. 16. Am. Soc. Rickettsiol. Rick. Dis. 12th Sesquiannual Mtg., Asilomar, Ca., March, 1996.
14. **Chen, S.-M., V. L. Popov, H.-M. Feng, J. Wen, and D. H. Walker.** 1995. Cultivation of *Ehrlichia chaffeensis* in mouse embryo, Vero, BGM, and L929 cells and study of *Ehrlichia*-induced cytopathic effect and plaque formation. Infect. Immun. **63**:647—653.
15. **Coughlin, R. T., C. Gingrich-Baker, T. Mather, and D. Fish.** 1996. Transmission, isolation, and cultivation of granulocytic *Ehrlichia* resulting from infection of dogs by adult *Ixodes scapularis* collected from eastern United States. Abstr. 52. Am. Soc. Rickettsiol. Rick. Dis. 12th Sesquiannual Mtg., Asilomar, Ca., March, 1996.
16. **Dasch, G. A., S. Halle, and A. L. Bourgeois.** 1979. Sensitive microplate enzyme-linked immunosorbent assay for detection of antibodies against the scrub typhus rickettsia, *Rickettsia tsutsugamushi*. J. Clin. Microbiol. **9**:38-48.
17. **Dasch, G. A., and E. Weiss.** 1977. Characterization of the Madrid E strain of *Rickettsia prowazekii* purified by Renografin density gradient centrifugation. Infect. Immun. **15**:280-286.
18. **Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. Goldsmith, K. H. Wilson, and C. W. Duntley.** 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. **29**:2741-2745.
19. **Dawson, J. E., D. B. Fishbein, T. R. Eng, M. A. Redus, and N. R. Greene.** 1990. Diagnosis of human ehrlichiosis with the indirect fluorescent antibody test: kinetics and specificity. J. Infect. Dis. **162**:91-95.
20. **Di Marco, A., M. Gaetani, P. Orezzi, B. M. Scarpinato, R. Silvestrini, M. Soldati, T. Dasdia, and L. Valentini.** 1964. "Daunomycin", a new antibiotic of the rhodomycin group. Nature **201**:706-707.
21. **Dimitriadis, G. J.** 1979. Effect of detergents on antibody-antigen interaction. Anal. Biochem. **98**:445-451.
22. **Dumler, J. S., K. M. Asanovich, J. E. Madigan, J. E. Barlough, J. S. Bakken, M. Aguero-Rosenfeld, G. P. Wormser, and J. L. Goodman.** 1996. Diagnosis of granulocytic ehrlichiosis by cell culture. Abstr. D-20. Am. Soc. Microbiol. Annual Mtg. , New Orleans, La., May, 1996.
23. **Dumler, J. S., K. M. Asanovich, J. E. Madigan, J. E. Barlough, J. S. Bakken, M.**

Aguero-Rosenfeld, G. P. Wormser, and J. L. Goodman. 1996. In vitro cultivation of the agents of human and equine granulocytic ehrlichiosis. Abstr. 55. Am. Soc. Rickettsiol. Rick. Dis. 12th Sesquiannual Mtg., Asilomar, Ca., March, 1996.

24. **Dumler, J. S. and J. S. Bakken.** 1995. Ehrlichial diseases of humans: emerging tick-borne infections. Clin. Infect. Dis. **20**:1102-1110.
25. **Dumler, J. S., J. S. Bakken, and K. M. Asanovich.** 1995. Human granulocytic ehrlichiosis in the upper midwest: the incidence of infection in febrile patients. Abstr. 95th General Meeting of the American Society for Microbiology, p. 272.
26. **Dumler, J. S., S.-M. Chen, K. Asanovich, E. Trigiani, V. L. Popov, and D. H. Walker.** 1995. Isolation and characterization of a new strain of *Ehrlichia chaffeensis* from a patient with nearly fatal monocytic ehrlichiosis. J. Clin. Microbiol. **33**:1704-1711.
27. **Fishbein, D. B., J. E. Dawson, and L. E. Robinson.** 1994. Human ehrlichiosis in the United States, 1985 to 1990. Annals Intern. Med. **120**:736-743.
28. **Goodman, J. L., C. Nelson, B. Vitale, J. E. Madigan, J. S. Dumler, T. J. Kurtti, and U. G. Munderloh.** 1996. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. New England J. Med. **334**:209—215.
29. **Hanson, B.** 1985. Identification and partial characterization of *Rickettsia tsutsugamushi* major protein immunogens. Infect. Immun. **50**:603-609.
30. **Hanson, B**. 1987. Factors influencing *Rickettsia tsutsugamushi* infection of cultured cells. Am. J. Trop. Med. Hyg. **36**:6212-630.
31. **Hanson, B.** Unpublished data.
32. **Hanson, B., and H. Paxton.** Unpublished data.
33. **Hanson, B., C. L. Wisseman, Jr., A. Waddell, and D. J. Silverman.** 1981. Some characteristics of heavy and light bands of *Rickettsia prowazekii* on Renografin gradients. Infect. Immun. **34**:596-604.
34. **Helenius, A., and K. Simons.** 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta **415**:29-79.
35. **Hilton, E., J. De Voti, J. S. Dumler, J. L. Benach, M. L. Halluska, D. J. White, and H. Paxton**. 1996. A study of seroprevalence and seroconversion for tick borne disease in a high risk population of the Northeast. Manuscript in preparation.
36. **Kardatzke, J. T., K. Neidhardt, D. P. Dzuban, J. L. Sanchez, and A. F. Azad.** 1992. Cluster of tick-borne infections at Fort Chaffee, Arkansas: rickettsiae and *Borrelia burgdorferi* in ixodid ticks. J. Med. Entomol. **29**:669-672.
37. **Kelly, D. J., C. T. Chan, H. Paxton, K. Thompson, R. Howard, ad G. A. Dasch.** 1995. Comparative evaluation of a commercial enzyme immunoassay for the detection of human antibody to *Rickettsia typhi*. Clin. Diag. Lab. Immunol. **2**:356-360.
38. **Koay, A. S., and Y. M. Cheong.** 1993. Preliminary evaluation of the INDX® DIP-

S-TICKS™ with positive rickettsial samples in Malaysia. Malaysian J. Pathol. **15**:69-73.

39. **Lugtenberg, B., and L. Van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737**:51-115.
40. **Madigan, J. E., P. J. Richter Jr., R. B. Kimsey, J. E. Barlough, J. S. Bakken, and J. S. Dumler.** 1995. Transmission and passage in horses of the agent of human granulocytic ehrlichiosis. J. Infect. Dis. **172**:1141-1144.
41. **Magnarelli, L. A., T. G. Andreadis, K. C. Stafford III, and C. J. Holland.** 1991. Rickettsiae and *Borrelia burgdorferi* in ixodid ticks. J. Clin. Microbiol. **29**:2798-2804.
42. **Magnarelli, L. A., J. S. Dumler, J. F. Anderson, R. C. Johnson, and E. Fikrig.** 1995. Coexistence of antibodies to tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in human sera. J. Clin. Microbiol. **33**:3054-3057.
43. **McDade, J. E.** 1990. Ehrlichiosis—a disease of animals and humans. J. Infect. Dis. **161**:609-617.
44. **McDade, J. E., and D. B. Fishbein.** 1988. *Rickettsiaceae*: the *Rickettsiae*, p. 864-890. *In* E. H. Lennette, P. Halonen, and F. A. Murphy (ed.), Laboratory diagnosis of infectious diseases, principles and practice, Vol. II. Viral, rickettsial, and chlamydial diseases. Springer-Verlag, New York.
45. **Moree, M. F., and B. Hanson.** 1992. Growth characteristics and proteins of plaque-purified strains of *Rickettsia tsutsugamushi*. Infect. Immun. **60**:3405-3415.
46. **Munderloh, U. G., J. E. Madigan, J. S. Dumler, J. L. Goodman, S. F. Hayes, J. E. Barlough, C. M. Nelson, and T. J. Kurtti.** 1996. Isolation of the equine granulocytic ehrlichiosis agent, *Ehrlichia equi*, in tick cell culture. J. Clin. Microbiol. **34**:664—670.
47. **Pancholi, P., C. P. Kolbert, P. D. Mitchell, K. D. Reed Jr, J. S. Dumler, J. S. Bakken, S. R. Telford 3rd, and D. H. Persing** . 1995. *Ixodes dammini* as a potential vector of human granulocytic ehrlichiosis. J. Infect. Dis. **172**:1007-12.
48. **Paparone, P. W., and W. B. Glenn.** 1994. Lyme disease with concurrent ehrlichiosis. J. Am. Osteopath. Assoc. **94**:568-577.
49. **Paxton, H.** Unpublished data.
50. **Perez, M., Y. Rikihisa, and B. Wen.** 1996. *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. J. Clin. Microbiol. **34**:2133-2139.
51. **Pradutkanchana, J., K. Silpapojakul, H. Paxton, S. Pradutkanchana, D. J. Kelly, and D. Strickman.** 1996. Comparative evaluation of four serodiagnostic tests for scrub typhus in Thailand. Submitted for publication.
52. **Pretzman, C., D. Ralph, D. R. Stothard, P. A. Fuerst, and Y. Rikihisa.** 1995. 16S rRNA gene sequence of *Neorickettsia helminthoeca* and its phylogenetic align-

ment with members of the genus *Ehrlichia*. Internatl. J. Syst. Bacteriol. **45**:207-211.

53. **Rikihisa, Y., S. A. Ewing, and J. C. Fox.** 1994. Western immunoblot analysis of *Ehrlichia chaffeensis, E. canis,* or *E. ewingii* infections in dogs and humans. J. Clin. Microbiol. **32**:2107-2112.
54. **Rikihisa, Y., N. Zhi, G. Wormser, B. Wen, H. W. Horowitz, and K. E. Hechemy.** 1996. Direct isolation and cultivation of human granulocytotropic ehrlichia from a human patient. Abstr. 54. Am. Soc. Rickettsiol. Rick. Dis. 12th Sesquiannual Mtg., Asilomar, Ca., March, 1996.
55. **Ristic, M.** 1990. Current strategies in research on ehrlichiosis, p. 136-153. *In* J. C. Williams and I. Kakoma (ed.). Ehrlichiosis. A vector-borne disease of animals and humans. Kluwer Academic Publishers, Boston.
56. **Schnaitman, C. A.** 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. **108**:545-552.
57. **Schnaitman, C. A.** 1971. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. J. Bacteriol. **108**:553-563.
58. **Shankarappa, B., S. K. Dutta, and B. L. Mattingly-Napier.** 1992. Antigenic and genomic relatedness among *Ehrlichia risticii, Ehrlichia sennetsu,* and *Ehrlichia canis*. Internatl. J. Syst. Bacteriol. **42**:127-132.
59. **Shea, K. W., A. J. Calio, N. C. Klein, and B. A. Cunha.** 1995. Rhabdomyolysis associated with *Ehrlichia chaffeensis* infection. Clin. Infect. Dis. **21**:1056-1057.
60. **Silpapojakul, K., J. Pradutkanchana, S. Pradutkanchana, and D. J. Kelly**. 1995 Rapid, simple serodiagnosis of murine typhus. Trans. Roy. Soc. Trop. Med. Hyg. **89**:625-628.
61. **Uhaa, I. J., J. D. Maclean, C. R. Greene, and D. B. Fishbein.** 1992. A case of human ehrlichiosis acquired in Mali: clinical and laboratory findings. Am. J. Trop. Med. Hyg. **46**:161-164.
62. **Walker, D. H., A. G. Barbour, J. H. Oliver, R. S. Lane, J. S. Dumler, D. T. Dennis, D. H. Persing , A. F. Azad, and E. McSweegan** . 1996. Emerging bacterial zoonotic and vector-borne diseases. Ecological and epidemiological factors. J. Am. Med. Assoc. **275**:463-469.
63. **Walker, D. H., and J. S. Dumler.** 1996. Emergence of the ehrlichioses as human health problems. Emerging Infect. Dis. **2**:18-29.
64. **Watt, G., D. Strickman, P. Kantipong, K. Jongsakul, and H. Paxton**. 1996. Prospective evaluation of a dotblot immunoassay for the rapid diagnosis of scrub typhus. Submitted for publication.
65. **Weddle, J. R., T.-C. Chan, K. Thompson, H. Paxton, D. J. Kelly, G. Dasch, and D. Strickman**. 1995. Effectiveness of a dot-blot immunoassay of anti-*Rickettsia tsutsugamushi* antibodies for serologic analysis of scrub typhus. Am. J. Trop.

Med. Hyg. **53**:43-46.

66. **Weiss, E., J. C. Coolbaugh, and J. C. Williams.** 1975. Separation of viable *Rickettsia typhi* from yolk sac and L cell host components by Renografin density gradient centrifugation. Appl. Microbiol. **30**:456-463.
67. **Weiss, E., G. A. Dasch, and Y. H. Kang.** 1985. Glutamine metabolism of *Ehrlichia sennetsu*, p. 38-45. *In* J. Kazar (ed.), Rickettsiae and rickettsial diseases. Slovak Academy of Science, Bratislava, Czechoslovakia.
68. **Weiss, E., G. A. Dasch, Y.-H. Kang, and H. N. Westfall.** 1988. Substrate utilization by *Ehrlichia sennetsu* and *Ehrlichia risticii* separated from host constituents by Renografin gradient centrifugation. J. Bacteriol. **170**:5012-5017.
69. **Weiss, E., J. C. Williams, G. A. Dasch, and Y.-H. Kang.** 1989. Energy metabolism of monocytic *Ehrlichia*. Proc. Natl. Acad. Sci. USA **86**:1674-1678.
70. **Wen B; Rikihisa Y; Mott J; Fuerst PA; Kawahara M; Suto C.** 1995. *Ehrlichia muris* sp. nov., identified on the basis of 16S rRNA base sequences and serological, morphological, and biological characteristics. Int J Syst Bacteriol 45(2):250-4.
71. **Wisseman, C. L. Jr., E. A. Edlinger, A. D. Waddell, and M. R. Jones.** 1976. Infection cycle of *Rickettsia rickettsii* in chicken embryo and L-929 cells in culture. Infect. Immun. **14**:1052-1064.
72. **Wisseman, C. L. Jr., A. D. Waddell, and D. J. Silverman.** 1976. In vitro studies on rickettsia-host cell interactions: Lag phase in intracellular growth cycle as a function of stage of growth of infecting *Rickettsia prowazekii* with preliminary observations on inhibition of rickettsial uptake by host cell fragments. Infect. Immun. **13**:1749-1760.
73. **Wong, S. J., R. A. Duncan, and G. S. Brady.** 1996. Lyme disease serology from human granulocytic ehrlichiosis cases from New York State. Abstr. 61. Am. Soc. Rickettsiol. Rick. Dis. 12th Sesquiannual Mtg., Asilomar, Ca., March, 1996.
74. **Woods, G. L., and D. H. Walker.** 1996. Chlamydial, rickettsial and mycoplasmal infections, p. 1115-1131. *In*, J. B. Henry (ed.), Clinical diagnosis and management by laboratory methods, W. B. Saunders Co., Philadelphia, Pa.
75. **Wu, S.-J. L., B. Hanson, H. Paxton, C. Rossi, E. A. Henchal, K. R. Porter, and C. G. Hayes.** 1996. Evaluation of a rapid dip-stick immunoassay for the detection of antibodies to dengue virus. Submitted for publication.
76. **Yevich, S. J., J. L. Sanchez, R. F. DeFraites, C. C. Rives, J. E. Dawson, I. J. Uhaa, B. J. Johnson, and D. B. Fishbein.** 1995. Seroepidemiology of infections due to spotted fever group rickettsiae and *Ehrlichia* species in military personnel exposed in areas of the United States where such infections are endemic. J. Infect. Dis. **171**:1266-73.

APPENDIX

TABLE 1. Comparison of human ehrlichial pathogens and selected other *Ehrlichia* species

EHRLICHIA	SIMILARITY[a]	REFERENCE
E. chaffeensis (*E. canis* group)		
E. canis	98.56	70
E. muris	98.92	70
E. sennetsu (*E. sennetsu* group)		
E. risticii	99.35	70
	99.36	52
HGE (*E. phagocytophila* group)		
E. equi	99.8	11
E. phagocytophila	99.9	11
VHE (*E. canis* group)		
E. canis	99.9	50

[a] Percent 16S rRNA gene sequence similarity

TABLE 2. Reactivity of ehrlichia antigen dipsticks with immune and nonimmune human sera.

SERUM[a]		NUMBER OF REACTIVE ANTIGEN DOTS[b]	
NUMBER	SPECIFICITY	*E. CHAFFEENSIS*	HGE
ECH-1	*E. chaffeensis*	1	2
ECH-2	*E. chaffeensis*	3	2
ECH-3	*E. chaffeensis*	2	3
ECH-4	*E. chaffeensis*	2	3
ECH-5	*E. chaffeensis*	2	3
ECH-6	*E. chaffeensis*	2	0
ECH-7	*E. chaffeensis*	1	0
EE-1	HGE	1	4
EE-2	HGE	2	2
EE-3	HGE	0	1
EE-4	HGE	0	2
EE-5	HGE	1	2
AL	normal	0	0
SB	normal	0	0
BG	normal	0	0
JW	normal	0	0

a Human sera were tested at 1:200 dilution in standard DIP-S-TICKS assay.

b Dipsticks were made with serial dilutions of antigen; the higher the number of reactive antigen dots, the greater the reaction with serum. Note that the two species of dipsticks contain different amounts of antigen and should not be compared with each other.

TABLE 3. Reactivity of ehrlichia antigen dipsticks with disease state and control human sera.

SERUM[a]		NUMBER OF REACTIVE ANTIGEN DOTS[b]	
NUMBER	SPECIFICITY	*E. CHAFFEENSIS*	HGE
RR-1	*Rickettsia rickettsii*	0	0
RC-1	*Rickettsia conorii*	0	4
RT-1	*Rickettsia typhi*	0	0
OTs-1	*Orientia tsutsugamushi*	0	0
Q-1	*Coxiella burnetii*	0	0
Lyme-1	*Borrelia burgdorferi*	0	2
DEN-JD	flavivirus	0	0
RPR +1	rapid plasma reagin	0	0
RPR +3	rapid plasma reagin	0	0
RPR +4	rapid plasma reagin	0	0
RNP-1	ribonucleoprotein	0	0
Smith-1	Smith antigen	0	0
DNA-1	DNA	0	1
ECH-6	*E. chaffeensis*	3	1
EE-1	HGE	0	4
AL	normal	0	0

a Human sera were tested at 1:200 dilution in standard DIP-S-TICKS assay.

b Dipsticks were made with serial dilutions of antigen; the higher the number of reactive antigen dots, the greater the reaction with serum. Note that the two species of dipsticks contain different amounts of antigen and should not be compared with each other.

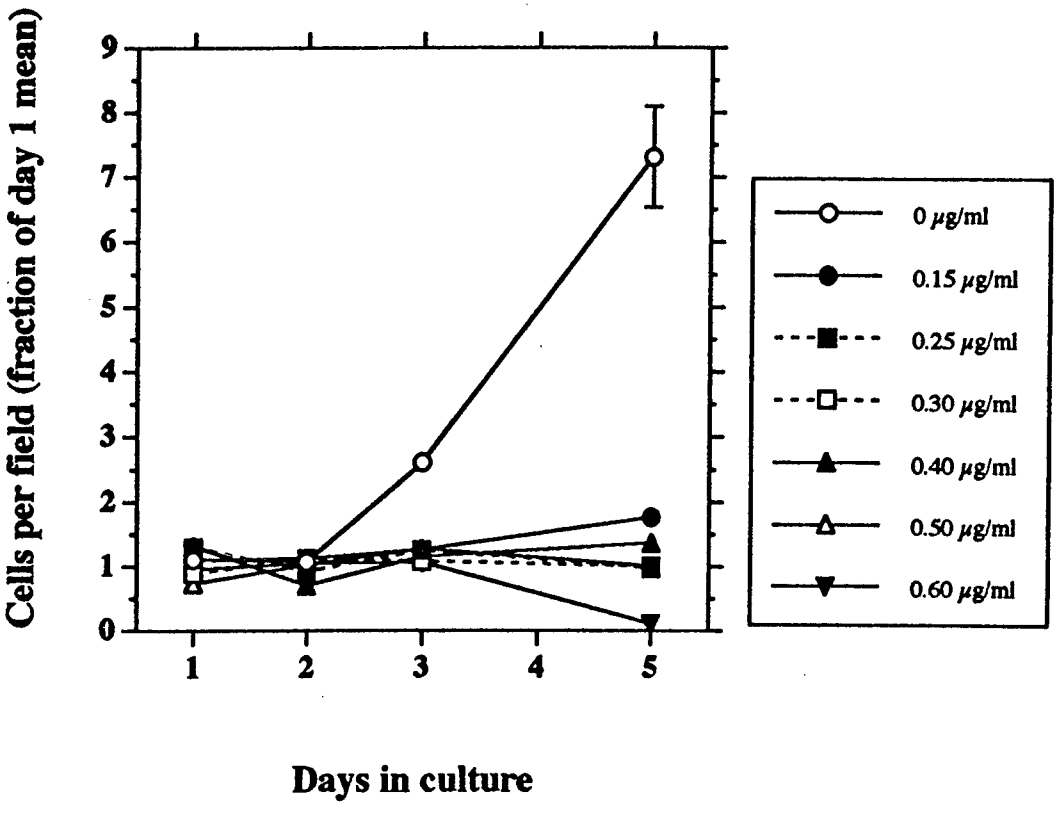

FIGURE 1. Effect of daunomycin on Vero cell replication. Vero cells were mixed with different concentrations of daunomycin and seeded into culture slides. Slides were fixed and stained at intervals and the mean number of cells per 1000X microscope field was calculated from quadruplicate counts of 25 fields each. Error bars are SE. Results show nontoxic inhibition with daunomycin at 0.25 — 0.5 μg per ml.

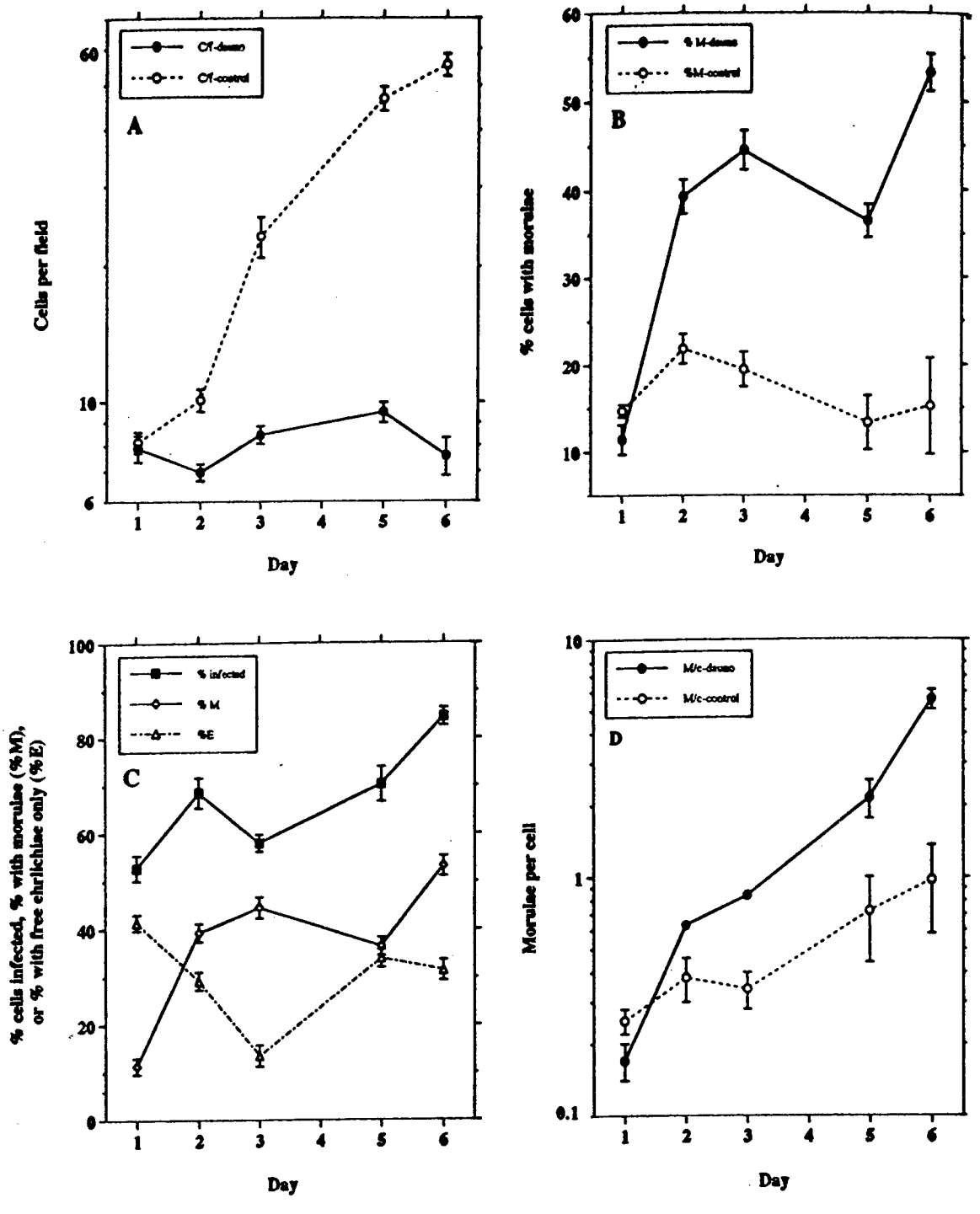

FIGURE 2. See following page for legend.

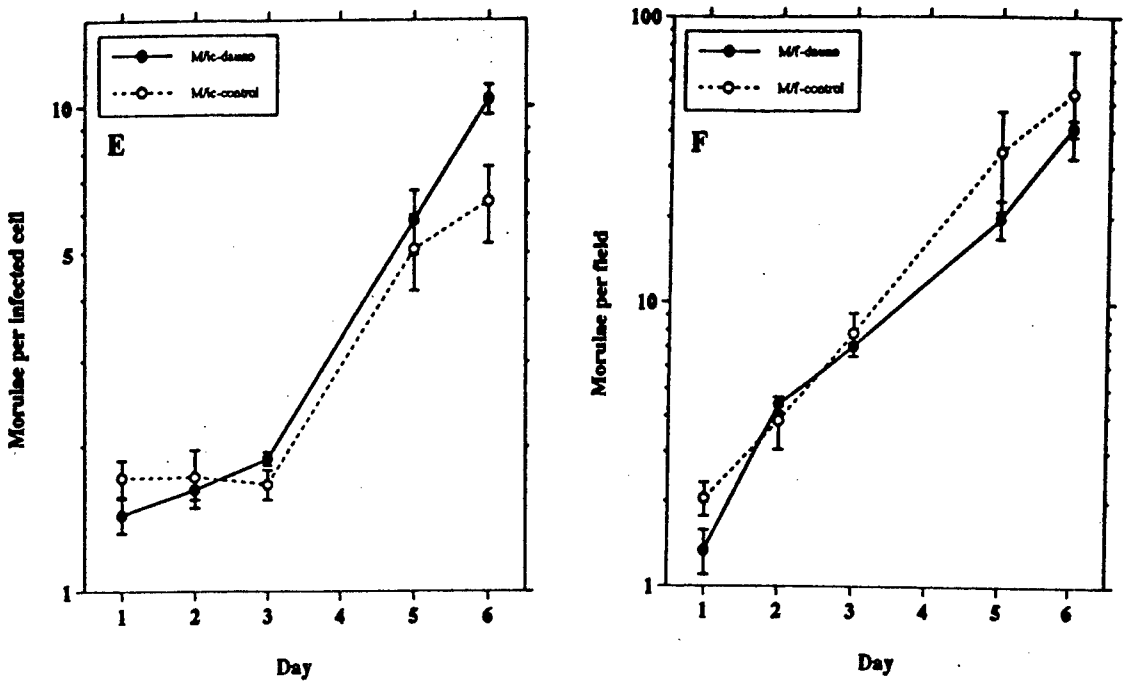

FIGURE 2. Effect of daunomycin on *E. chaffeensis* replication in Vero cells. Vero cells were infected in suspension and then seeded into culture slides in the presence (0.25 and 0.40 μg per ml results were similar and thus combined) or absence of daunomycin. Slides were fixed daily for enumeration of cells per 1000X microscope field and ehrlichia replication. Vero cell counts: 8 or 4 replicate counts of 25 random microscope fields each for treated or untreated cells, respectively. Ehrlichia counts: 6 or 3 replicate counts of 100 cells each for treated or untreated cells, respectively. Error bars are SE. **A.** Vero cell replication. **B.** Percent cells containing morulae. (They may or may not also contain free ehrlichiae.) **C.** Infection in daunomycin-treated cells only; percent infected is percent M + percent E. **D.** Mean number of morulae per cell. **E.** Mean number of morulae per infected cell. **F.** Mean number of morulae per field: each replicate count of mean number of morulae per cell was multiplied by mean number of cells per field.

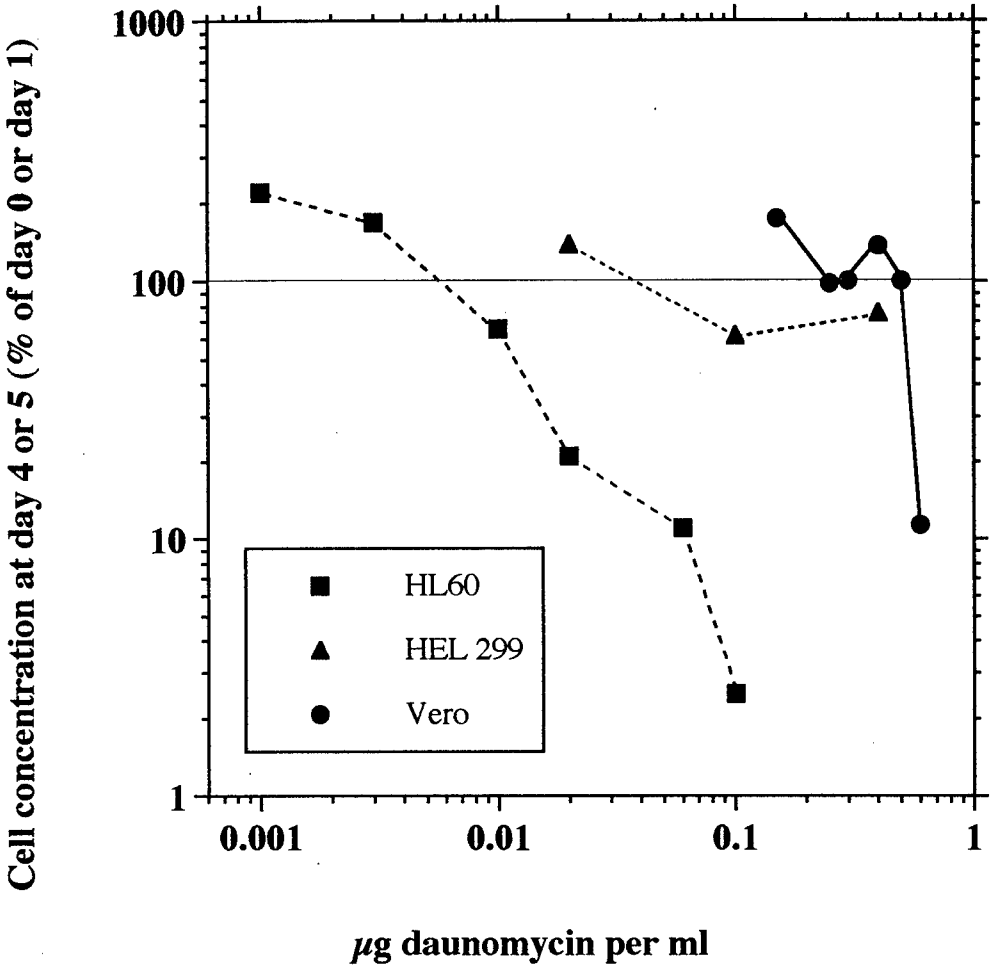

FIGURE 3. Effect of daunomycin on HL-60, HEL 299, and Vero cell replication. Cells were treated on day 0 with different concentrations of daunomycin and were counted daily. Results are presented as mean number of cells per ml on day 4 (HL-60) or per 1000X microscope field on day 5 (HEL 299 and Vero), given as percent of the cell counts on day 0 (HL-60) or day 1 (HEL 299 and Vero). Untreated control values on day 4 or 5 were: HL-60 — 230%; HEL 299 — 630%; Vero — 740%.

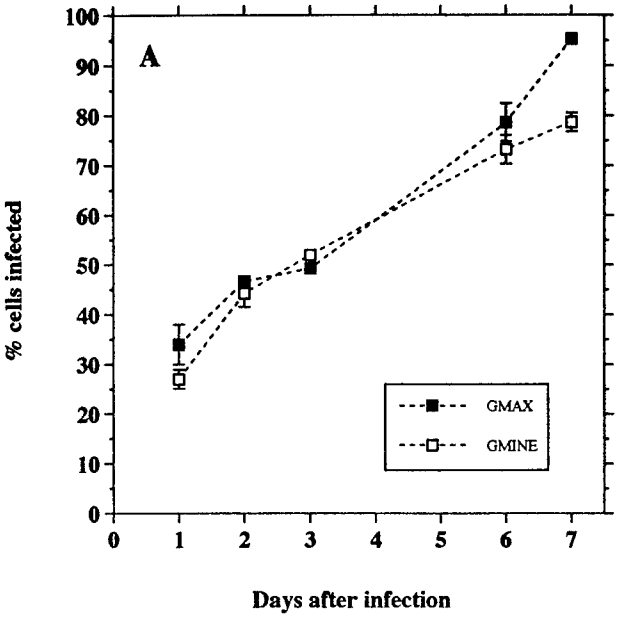

FIGURE 4. Comparison of glutamine (GMINE) and Glutamax (GMAX; L-alanyl-L-glutamine) in support of *E. chaffeensis* replication in daunomycin-inhibited Vero cells. Medium was RPMI 1640 containing 0.2% sodium bicarbonate, 5% fetal bovine serum, and either 2 mM Glutamax or 2 mM L-glutamine. Slide cultures were grown at 35°C in an atmosphere of 5% CO_2 in air and were fed with fresh medium on day 6. Bars are SE. **A.** Percent cells infected (morulae or individual ehrlichiae). Each point represents the mean of 3 counts of 100 cells each.

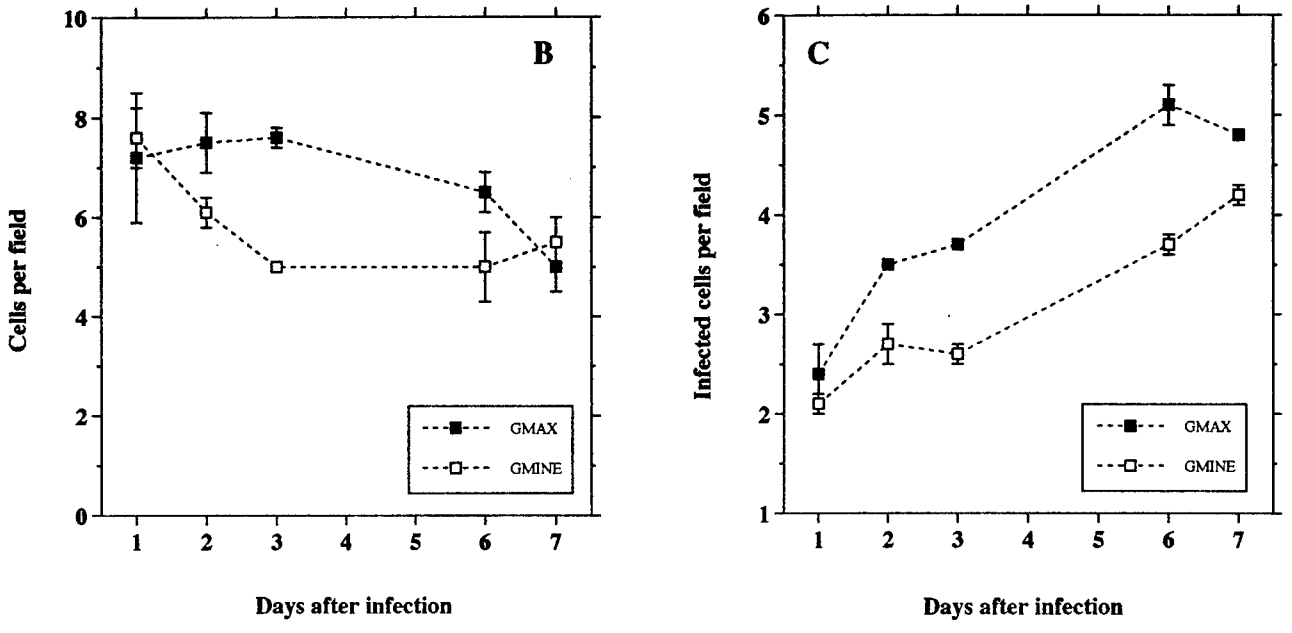

FIGURE 4. (*continued*) **B.** Mean number of cells per field. Each point is from 4 counts of 25 1000X microscope fields. **C.** Mean number of infected cells per 1000X microscope field: fraction of cells infected (triplicate counts of 100 cells) times mean number of cells per field.

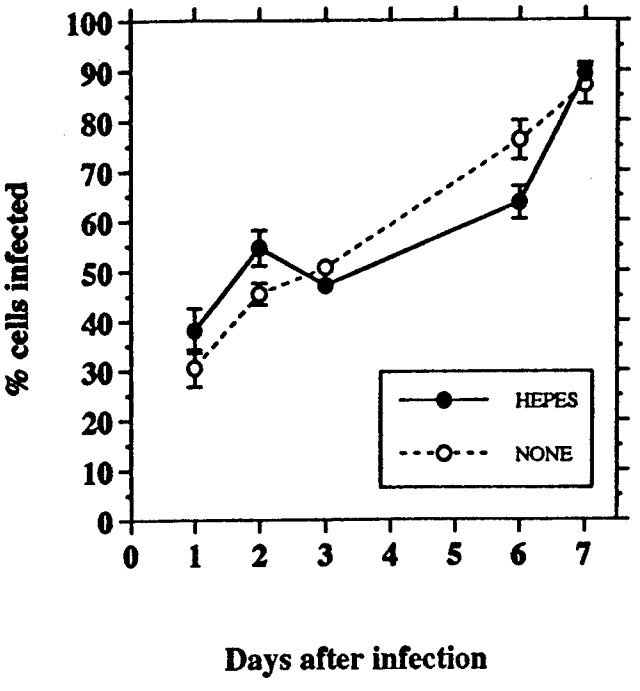

FIGURE 5. Effect of HEPES buffer on the replication of *E. chaffeensis* in daunomycin -inhibited Vero cells. Medium was RPMI 1640 containing 0.2% sodium bicarbonate, 5% fetal bovine serum, either 2 mM Glutamax or 2 mM L-glutamine, and with or without 15 mM HEPES. Slide cultures were grown at 35°C in an atmosphere of 5% CO_2 in air and were fed with fresh medium on day 6. Each point represents the mean percent cells infected (morulae or individual ehrlichiae) based on 6 counts of 100 cells each. Bars are SE.

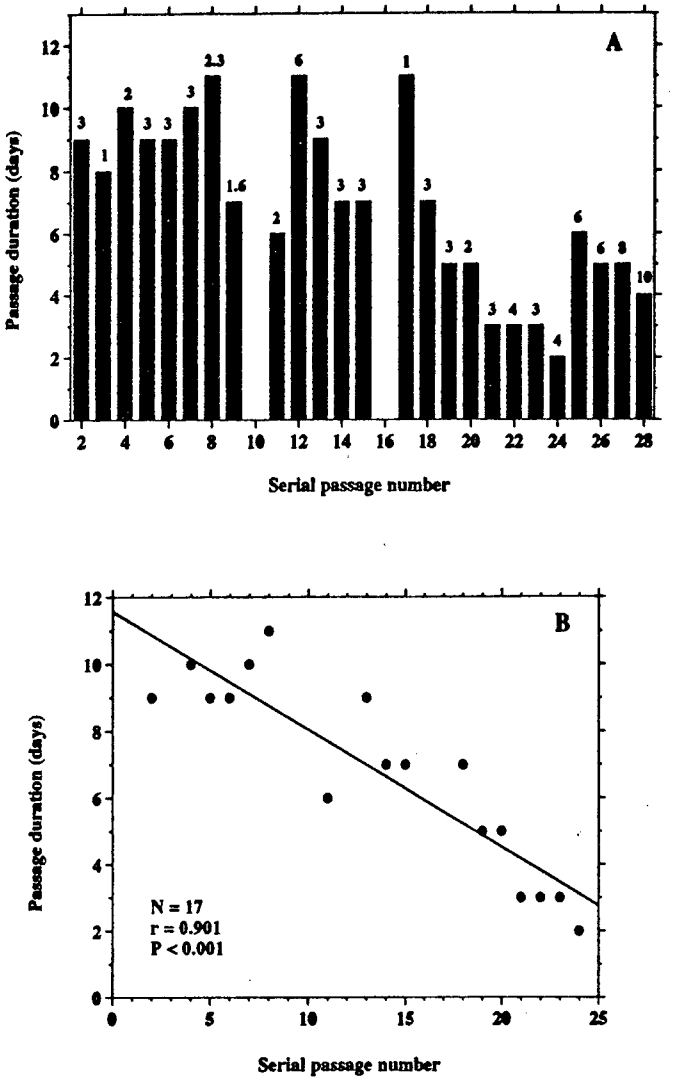

FIGURE 6. Serial passage of *E. chaffeensis* in daunomycin-inhibited Vero cells. The passage duration (days after infection that the cultures were passaged) was determined by CPE and extent of infection observed in Giemsa-stained cultures. A. Cells were infected at the split ratios (uninfected flasks made from each infected flask) indicated above each bar. Ehrlichiae were frozen after the 9th and 15th passages; the passage times after infection with frozen stocks were considerably longer than with unfrozen organisms and are not shown. B. Cultures that were infected at a split ratio of 2-4 to 1 (uninfected to infected flasks) are represented.

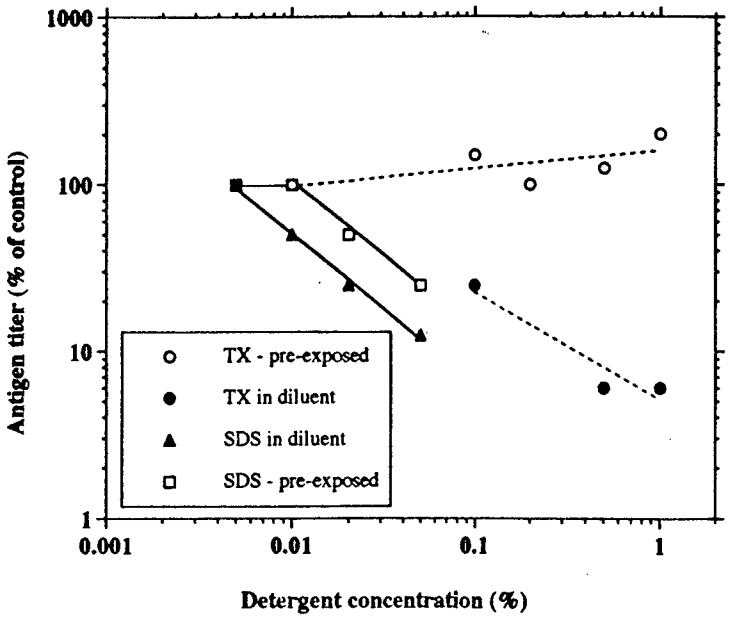

FIGURE 7. Effect of TX or SDS on the antigenicity of Renografin-purified *E. chaffeensis*. Ehrlichiae were either diluted in buffer containing the indicated detergent concentrations or were preexposed to detergent and then serially diluted in PBS. Antigen was applied to DS and tested with specific antiserum.

BIBLIOGRAPHY

No publications or meeting abstracts have resulted from this work yet.

PERSONNEL

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Helene Paxton (Integrated Diagnostics)
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