The Role of Mononuclear Phagocytes in HTLV-III/LAV Infection

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Cells with properties characteristic of mononuclear phagocytes were evaluated for infectivity with five different isolates of the AIDS virus, HTLV-III/LAV. Mononuclear phagocytes cultured from brain and lung tissues of AIDS patients harbored the virus. In vitro–infected macrophages from the peripheral blood, bone marow, or cord blood of healthy donors produced large quantities of virus. Virus production persisted for at least 40 days and was not dependent on host cell proliferation. Giant multinucleated cells were frequently observed in the macrophage cultures and numerous virus particles, often located within vacuole-like structures, were present in infected cells. The different virus isolates were compared for their ability to infect macrophages and T cells. Isolates from lung- and brain-derived macrophages had a significantly higher ability to infect macrophages than T cells. In contrast, the prototype HTLV-III$_B$ showed a 10,000-fold lower ability to infect macrophages than T cells and virus production was one-tenth that in macrophage cultures infected with other isolates, indicating that a particular variant of HTLV-III/LAV may have a preferential tropism for macrophages or T cells. These results suggest that mononuclear phagocytes may serve as primary targets for infection and agents for virus dissemination and that these virus-infected cells may play a role in the pathogenesis of the disease.

HTLV-III/LAV, THE ETIOLOGIC agent of AIDS (*1*), is lymphotropic for the helper/inducer subset of T4$^+$ lymphocytes (*2*). Although selective depletion of these T cells may explain some aspects of the immunodeficiency that develops in AIDS patients, it cannot account for certain other clinical features of the disease. There is evidence to suggest that cells other than T lymphocytes can be infected by the virus. It has been demonstrated in vitro that some rare B lymphocytes and a neoplastic cell line of monocyte-macrophage origin, both of which express T4 antigen, can be infected with HTLV-III/LAV (*3*). Central nervous system disease, characterized by the development of an unexplained generalized encephalopathy associated with progressive dementia, is often seen in patients with AIDS (*4*). HTLV-III/LAV sequences within the host cell DNA and HTLV-III/LAV messenger RNA have been detected in brain tissues recovered from neurosymptomatic AIDS patients by Southern blot analysis and in situ hybridization, respectively (*5*). It was concluded from these studies that the cells harboring the virus in the brain were not T lymphocytes. Characterization of primary cultures of brain tissues from an AIDS patient showed that the virus-positive cells belonged to the mononuclear phagocyte series (*6*). Moreover, at an early stage of HTLV-III/LAV infection, as seen in patients with persistent generalized lymphadenopathy (PGL), the germinal centers within the lymph nodes are characterized by an expansion of follicular dendritic (FD) cells and increased numbers of B blast cells. The presence of retrovirus particles, as well as the expression of HTLV-III/LAV core proteins, have been demonstrated within the network of these FD cells (*7*). It is conceivable that the defect in antigen presentation that has been observed in AIDS patients (*8*) may be a consequence of virus infection of macrophages. However, the direct infection of cells of the mononuclear phagocyte series with HTLV-III/LAV has not been clearly shown.

We have recovered macrophages from a variety of tissues and maintained them in vitro for several months using methods previously described (*9*). An essential step in this procedure is the enhancement of early adherence of these cells to the surface of plastic flasks by adding 10% heat-inactivated, HTLV-III/LAV antibody–negative, pooled human serum to the culture medium. Three to five days after the initiation of the cultures, the macrophage monolayers are washed extensively with phosphate-buffered saline (PBS) to ensure the removal of all nonadherent cells. By this method, we obtained pure populations of macrophages that could be infected with HTLV-III/LAV (Fig. 1A). Cultures established in a similar fashion without the addition of human serum were also susceptible to and permissive for HTLV-III/LAV. However, 7 days was usually required to obtain adequate monolayers of macrophages when human serum was not included in the medium.

The plastic-adherent macrophage cultures were trypsin-resistant and up to 100% positive for nonspecific esterase (NSE) (Fig. 1B and Table 1). Additionally, over 95% of the cells exhibited phagocytic activity as demonstrated by the ingestion of latex beads (Fig. 1C). Indirect immunofluorescence (IF) studies with the use of monoclonal antibodies provided further evidence that these cell cultures represented pure populations of macrophages. On the average, the cultures were 90% OKM1-positive (Ortho Diag-

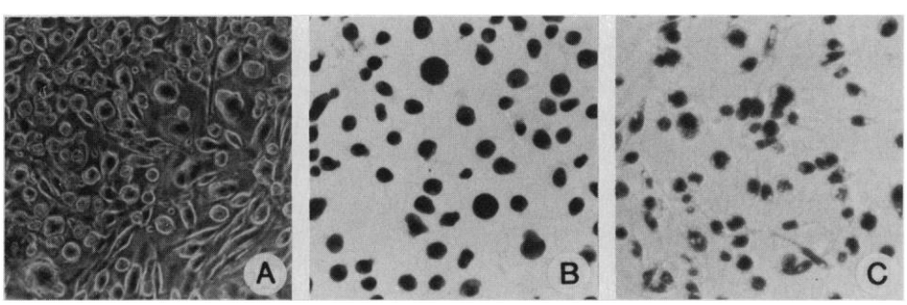

Fig. 1. Characteristics of macrophages derived from the peripheral blood of a healthy donor: (A) growth pattern (×220), (B) nonspecific esterase (NSE) staining (×260), and (C) phagocytosis of latex beads (×200). Cultures of pure populations of macrophages were obtained as described (*9*). Briefly, 3×10^7 mononuclear cells recovered from Ficoll-Hypaque gradients were seeded into T25 plastic flasks (Corning) in RPMI 1640 supplemented with antibiotics, 20% heat-inactivated FBS and 10% heat-inactivated pooled human serum (Advanced Biotechnologies) negative for HTLV-III antibodies as determined by ELISA. To remove nonadherent cells, cultures were washed extensively with phosphate buffered saline 3 to 5 days after initiation of the cultures. Macrophage cultures were routinely maintained in RPMI 1640 supplemented with antibiotics and 20% heat-inactivated FBS. Cytochemical staining of cell cultures for α-naphthyl acetate esterase (nonspecific esterase) was performed on cells fixed in situ with Sigma kit No. 90. Phagocytic activity was demonstrated by incubating macrophage cultures at 37°C for 24 hours in the presence of 1.05-μm latex beads (Polysciences). After the incubation period the cultures were washed with RPMI 1640 medium to remove the excess beads and examined microscopically.

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nostics), 80% Leu-M5–positive (Becton-Dickinson), and 70% MAC-1–positive (Hybritech). No staining was observed with the monoclonal antibodies OKT3, OKT4, or OKB7 (Ortho). However, approximately 33% of the cells reacted with OKT4a (Ortho), indicating the expression of the T4 antigen by these macrophages. Because of the consistent positive correlation between trypsin-resistant plastic adherence and NSE positivity compared to staining with antibodies specific for macrophages, we concluded that the former properties represent reliable markers of macrophages. Trypsin-resistant plastic adherence and NSE positivity proved to be more easily and accurately applied than staining with specific antibodies to primary cultures of tissues such as lung and brain where the majority of cells were not members of the mononuclear phagocyte series.

Five different HTLV-III/LAV isolates were used in the transmission studies. Two isolates, designated HTLV-$III_{RC\text{-}br}$ and HTLV-$III_{RC\text{-}PB}$, were obtained from the same patient. The former was isolated from primary cultures of brain tissue containing cells with mononuclear phagocyte characteristics. The latter was recovered from cultured T cells of the patient's peripheral blood (PB) (*6*). Two isolates, HTLV-III_{MN} and the prototype HTLV-III_B, were recovered from T cells of PB from patients with AIDS (*1*). The fifth isolate, HTLV-$III_{Ba\text{-}L}$, was isolated from primary cultures of lung tissue from a child with AIDS.

Only primary cultures of brain and lung tissues containing cells that were NSE-positive and not susceptible to trypsinization expressed the virus (Table 1). Subcultured trypsin-sensitive cells from these brain and lung cultures were negative for NSE and did not express the virus. Attempts to infect these subcultured NSE-negative cells with two different isolates, HTLV-III_B and HTLV-$III_{RC\text{-}br}$, gave consistently negative results. On the contrary, infection of mononuclear phagocytes originating from 25 different specimens of PB from 20 different donors, and from one sample of cord blood and one of bone marrow, were highly susceptible to and permissive for the virus. The percentages of HTLV-III/LAV–infected cells, as determined by IF assay with the use of highly specific mouse monoclonal antibodies recognizing the viral core protein HTLV-III p17, ranged from 5% to 21% in macrophage cultures derived from PB (*10*).

Virus production was detected in culture fluids by reverse transcriptase (RT) assay (*11*) and ranged from 20,000 to 800,000 cpm/ml. In some experiments, with the brain- or lung-derived isolates, RT activity in macrophage cultures reached 2 million counts per minute per milliliter of culture fluid. These results indicate that mononuclear phagocytes from different tissues are susceptible to and permissive for various HTLV-III/LAV isolates.

To substantiate further that HTLV-III/LAV replicates in macrophages, we performed DNA hybridizations using samples of DNA from these cells as well as from T lymphocytes, both of which were infected with the brain isolate HTLV-$III_{RC\text{-}br}$, and from macrophages infected with an isolate from lung tissue, HTLV-$III_{Ba\text{-}L}$. Both the isolates used in these studies were recovered from cells of the mononuclear phagocyte series from AIDS patients and had been propagated in vitro only on PB-derived macrophages. The DNA samples were digested separately with several restriction enzymes. Undigested DNA was also examined. Figure 2 depicts a representative Southern blot of a hybridization analysis obtained by Hind III digestion. A comparison of lanes c and d reveals that the brain-derived (HTLV-$III_{RC\text{-}br}$) isolate transmitted to PB-derived macrophages is identical to that transmitted into T lymphocytes. Lane f contained the HTLV-$III_{Ba\text{-}L}$ isolate that showed a restriction pattern different from the brain-derived isolate. A comparison of lane a with lanes c, d, and f reveals that the HTLV-$III_{RC\text{-}br}$ and HTLV-$III_{Ba\text{-}L}$ isolates are clearly distinct from the prototype HTLV-III_B commonly used in our laboratory. In addition, as the biological assays indicated, subcultured brain and lung cells from the primary cultures established from AIDS patients' tissues contained no detectable HTLV-III/LAV sequences (Fig. 2, lanes e and g).

Virus-infected macrophage cultures frequently contained giant multinucleated cells, some cells having more than 100 nuclei (Fig. 3, A and B). In general, the virus-induced cytopathic effect on these cultured macrophages was not as profound as that in cultures of virus-infected T cells (*1, 2*). Electron microscopic examinations of the

Table 1. Infection of mononuclear phagocytes with isolates of HTLV-III/LAV. The HTLV-$III_{RC\text{-}br}$ isolate was recovered from adherent cells of primary brain cultures grown in Dulbecco's Hi-glucose minimum essential medium (MEM) supplemented with 15% fetal bovine serum (FBS) and antibiotics. HTLV-$III_{RC\text{-}PB}$ was recovered from the same patient's peripheral blood (PB) T cells and propagated in PB-derived T cells as previously described (*1*). These isolates were obtained from a 50-year-old bisexual male with neurological symptoms who subsequently developed AIDS (*6*). The prototype HTLV-III_B and the HTLV-III_{MN} isolate were propagated in H9 cells (*1*). The HTLV-$III_{Ba\text{-}L}$ isolate was recovered from primary lung cultures grown in RPMI 1640 supplemented with 20% FBS and antibiotics (complete culture medium). The postmortem specimen had been obtained from the lung of a 7-month-old boy who died of AIDS. HTLV-$III_{RC\text{-}br}$ and HTLV-$III_{Ba\text{-}L}$ were propagated in PB-derived macrophages. For macrophage culture conditions, see legend to Fig. 1. Details of the infection procedures are described (*23*). Cultures were subjected to trypsinization by exposing the adherent cells to Ca^{2+}-Mg^{2+}–free PBS containing 0.05% trypsin (Gibco) and 0.05% EDTA for 1 to 5 minutes at 37°C. Flasks still containing cells were re-fed with complete culture medium. See legend to Fig. 1 for NSE staining. Macrophages were prepared for immunofluorescence assay as described (*24*). Highly specific mouse monoclonal antibody against the viral core protein HTLV-III p17 was developed by standard procedures (*10*). Details regarding the RT assays are described (*11, 25*). NT, not tested.

Virus isolate and source of tissue	Samples virus-positive/samples analyzed	Characteristics of cultured cells		Virus expression	
		Response to trypsin	Number NSE-positive (%)	HTLV-III p17 (% positive)	RT activity (cpm/ml)
HTLV-$III_{RC\text{-}br}$					
Brain (primary)	1/1	Resistant	1	NT	2×10^4
Brain (subcultured)	0/1	Sensitive	0	0	0
Skin (subcultured)	0/1	Sensitive	0	0	0
Peripheral blood	11/11	Resistant	>95	5 to 21	0.02×10^6 to 2×10^6
HTLV-$III_{RC\text{-}PB}$					
Peripheral blood	3/3	Resistant	>95	14	0.24×10^6 to 1.3×10^6
HTLV-III_B					
Peripheral blood	3/3	Resistant	>95	5 to 10	0.3×10^5 to 1×10^5
Bone marrow	1/1	Resistant	20	1	4×10^4
Bone marrow (subcultured)	0/1	Sensitive	0	0	0
HTLV-III_{MN}					
Cord blood	1/1	Resistant	>95	NT	3.7×10^4
HTLV-$III_{Ba\text{-}L}$					
Lung (primary)	1/1	Resistant	10	NT	0.65×10^4
Lung (subcultured)	0/1	Sensitive	0	0	0
Peripheral blood	8/8	Resistant	>95	10 to 20	1.3×10^6 to 2×10^6

infected macrophage cultures revealed the presence of abundant numbers of virus particles which, as demonstrated by staining with ruthenium red (*12*), were frequently located within vacuole-like structures (Fig. 3C).

To determine whether cell proliferation is required for the continuous production of HTLV-III/LAV by macrophages infected with the virus, we evaluated the mitotic index (MI) in these cultures. They were exposed for 28 hours to 0.1 μg of Colcemid per milliliter of culture fluid. Culture fluids were harvested immediately prior to the addition of the Colcemid and again at the end of the 28-hour exposure period. The adherent cells were fixed and stained with Wright's-Giemsa in situ. Those few macrophages that became detached from the flasks during the 28 hours were recovered and also examined. The number of cells in mitosis per flask was on the average 25 per 10^6 cells (MI = 0.000025) during the 28-hour interval, indicating that cell proliferation was negligible and that the cultures were stationary cell populations. Virus production before and during the 28-hour interval essentially did not change and was on the average 45,000 cpm per milliliter of culture fluid as determined by RT assay (*11*). These results show that virus replication in HTLV-III/LAV–infected macrophages is independent of cell proliferation. Presumably, this is because virus replication in these cells can involve unintegrated copies of the viral DNA, a phenomenon that was first demonstrated in spleen necrosis virus–infected cells (*13*). Furthermore, Southern blot analysis of DNA recovered from HTLV-III/LAV–infected macrophages revealed that, as previously demonstrated in the case of HTLV-III/LAV–infected T lymphocytes (*14, 15*), a significant amount of the viral DNA was present in an unintegrated form.

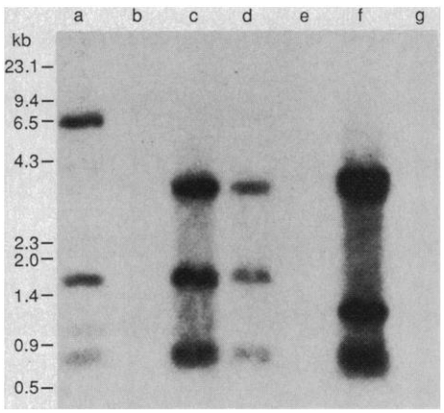

Fig. 2. Southern blot analysis of Hind III–digested DNA from: (a) H9/HTLV-III_B cells; (b) H9 uninfected cells; (c) normal peripheral blood (PB)–derived macrophages infected with HTLV-$III_{RC\text{-}br}$; (d) normal PHA-stimulated IL-2–dependent T lymphocytes infected with HTLV-$III_{RC\text{-}br}$; (e) subcultured glial-like cells derived from the same brain biopsy from which HTLV-$III_{RC\text{-}br}$ was isolated; (f) normal PB-derived macrophages infected with HTLV-$III_{Ba\text{-}L}$; and (g) subcultured fibroblast-like cells derived from the same lung specimen from which HTLV-$III_{Ba\text{-}L}$ was isolated. As shown, the restriction patterns of the viral isolates HTLV-$III_{RC\text{-}br}$ and HTLV-$III_{Ba\text{-}L}$ are different from each other and from the prototype HTLV-III_B. No HTLV-III/LAV sequences were detected in the trypsin-sensitive subcultured brain and lung cells. The experimental conditions have been described (*26*). The major internal restriction fragments generated by Hind III digestion of H9/HTLV-III_B (lane a) were 6.5, 4.4, 1.8, and 0.7 kb in length. Those generated by Hind III digestion of DNA from macrophages and T cells infected with HTLV-$III_{RC\text{-}br}$ (lanes c and d) were 4.1, 1.8, and 0.8 kb in length, and those generated by Hind III digestion of DNA from HTLV-$III_{Ba\text{-}L}$–infected macrophages (lane f) were 4.1, 1.2, and 0.8 kb in length.

We then compared quantitatively the susceptibility of macrophages and T cells to HTLV-III/LAV using cells derived from the PB of healthy donors. Approximately equal numbers of both types of cells (2×10^6) from the same donor were exposed to serial or tenfold dilutions of the virus inoculum. In the usual fashion, the T lymphocytes were stimulated with phytohemagglutinin (PHA) prior to infection and maintained in medium containing exogenous T cell growth factor (TCGF, IL-2). Virus production was measured in culture fluids harvested from these cultures at 3-day intervals for 1.5 months. The experiments were terminated when, in three successive RT assays, no further increase in virus release could be detected. As shown in Table 2, macrophages and T cells showed the same susceptibility to infection with the T cell–derived isolate HTLV-$III_{RC\text{-}PB}$ when the T cells were infected immediately after PHA stimulation, while T cell susceptibility to this isolate decreased tenfold when these T cells were infected after having been maintained for 6 days in IL-2–containing medium. It is possible that this tenfold difference was a consequence of the presence of some macrophages in those T cell cultures infected immediately after PHA stimulation. The isolates HTLV-$III_{RC\text{-}br}$ and HTLV-$III_{Ba\text{-}L}$, which were both recovered from cells of the mononuclear phagocyte series, were 10- to 100-fold more efficient in infecting macrophages than T cells. In contrast, the prototype HTLV-III_B, which has been propagated for a long time only in T cells, exhibited approximately a 10,000-fold higher efficiency in infecting T cells than macrophages. The fact that macrophages were clearly more susceptible to the HTLV-$III_{RC\text{-}br}$ and HTLV-$III_{Ba\text{-}L}$ isolates whereas T cells were more susceptible to the prototype isolate HTLV-III_B suggests that the T cell tropism of the virus may be an acquired behavior resulting from the continual propagation of the virus in $T4^+$ cells both in vitro and in vivo.

The permissivity of macrophages and T cells to infection with HTLV-III/LAV was compared quantitatively again with the use of autologous cells derived from the PB of healthy donors. Approximately equal numbers of autologous macrophages and T cells (2×10^6) were exposed to the same virus inoculum (within the range of 10,000 to 100,000 cpm/ml) of the HTLV-$III_{RC\text{-}br}$ and HTLV-$III_{Ba\text{-}L}$ isolates. These isolates had been propagated previously only on macrophages. The longevity and magnitude of virus production were followed in culture fluids by RT assay at 3- to 5-day intervals for 2 to 4 months. A representative example of the results of these experiments is shown in Fig. 4. The peak of virus production in HTLV-$III_{RC\text{-}br}$–infected macrophage cultures was tenfold higher than in the infected

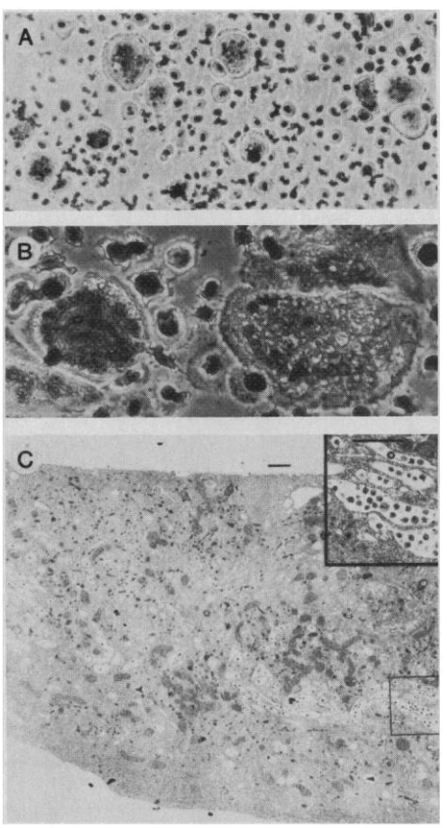

Fig. 3. (A and B) Morphology of HTLV-$III_{RC\text{-}br}$–infected PB-derived macrophages stained with Wright's-Giemsa 2 weeks after infection (A, ×80; B, ×640). Note the presence of numerous multinucleated giant cells. (C) Electron micrograph depicting a macrophage infected with HTLV-$III_{RC\text{-}br}$. The section was taken from a culture fixed in situ. That part of the cell adhering to the plastic flask is located at the top of the photograph. The inset shows an abundance of virus particles located within vacuole-like structures (scale bar, 1 μm).

T cell cultures, and production persisted for at least 40 days longer. Although the numbers of cells exposed to the virus were comparable, it should be noted that the macrophage cultures were essentially static, nondividing cell populations, while the T cells, maintained in IL-2, continued to proliferate, resulting in substantial increases in the total number of cells. At the peak points of virus production shown in Fig. 4, 10% of the macrophages and 6% of the T cells were HTLV-III p17–positive, which was equal to approximately 25,000 macrophages and 60,000 T cells per milliliter of culture fluid, respectively. Therefore, the difference in virus production between macrophages and T cell cultures was even greater if expressed on a per cell basis. All five isolates used in these studies showed essentially the same pattern of long-term virus production in macrophage cultures and transient production in T cell cultures. There were, however, some differences in the magnitude of virus production by macrophages among different isolates. For instance, under the same culture conditions, virus production by HTLV-III_B–infected macrophages was one-tenth the amount that HTLV-III_{Ba-L}–infected macrophage cultures produced (see Table 1).

These results indicate that cells of the mononuclear phagocyte series recovered from brain and lung tissues of AIDS patients harbored the virus. Since it is well established that the first event that occurs when a foreign material (for example, virus) enters the body is its uptake by mononuclear phagocytes, it is conceivable that these cells represent a primary target for the virus. In addition, virus production by HTLV-III/LAV–infected macrophages was high and long-lived, indicating that these cells may play a role in virus dissemination and persistence. Several lines of evidence, including nucleotide sequence homology (*16, 17*) and morphology of the virus particles (*17*), suggest a relation between visna virus, a lentivirus, and HTLV-III/LAV. The findings presented here provide additional evidence of a similarity, since macrophages are the major targets for visna virus (*18*). However, visna virus replication in macrophages has been shown to be minimally productive (*19*) while HTLV-III/LAV replication in macrophages was fully productive in the situations we evaluated. Also, the presence of 3′orf (*20*) and the ability to acquire an affinity to T lymphocytes appear to be features unique to HTLV-III/LAV.

The prototype HTLV-III_B, which can infect macrophages, has been selected for growth on normal and neoplastic T cells. Quantitative titration of this isolate on T cells and macrophages showed a 10,000-fold greater susceptibility of $OKT4^+$ T cells than macrophages. Moreover, a binding assay revealed high-affinity binding of the virus particles to the T4 antigen of $T4^+$ cells (*21*), suggesting that, through infection and growth in $T4^+$ cells, HTLV-III/LAV can increase its tropism to $T4^+$ cells. Since $T4^+$ helper cells are the first type of cells that interact with macrophages after antigen uptake, it is probable that HTLV-III/LAV infection in vivo initially follows the same general pathway as any other antigen that enters the human body. However, because the virus can acquire a high affinity for and also chronically infect T lymphocytes (*22*), it is likely that transmission in vivo from T cells to T cells and from T cells to macrophages also occurs. It is also conceivable that the ability of the virus to develop into a particular variant with preferential tropism for macrophages or T cells may partly determine both the appearance of certain symptoms and the clinical course of the disease.

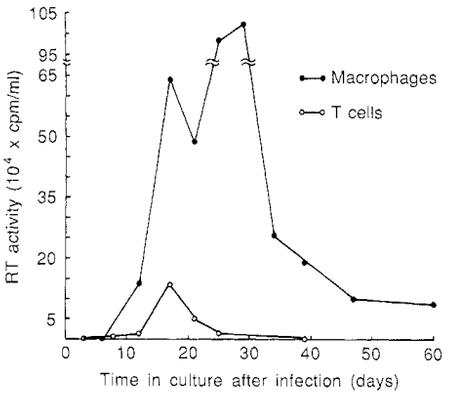

Fig. 4. Comparison of HTLV-III_{RC-br} production by macrophages and T cells during long-term cultivation in vitro. Approximately 2×10^6 macrophages and T cells from the same donor were exposed separately to 1 ml of virus inoculum containing 10,000 cpm of RT activity for 60 minutes as previously described (see legend to Table 1). Culture fluids were harvested at 3- to 5-day intervals and assayed for RT activity (*11, 25*). At the peak points of virus production, 10% of macrophages and 6% of T cells were HTLV-III p17–positive as determined by IF assay (*10, 24*). The values are expressed as counts per minute per milliliter of culture fluid.

Table 2. Relative susceptibility of macrophages and T cells to infection with different HTLV-III/LAV isolates in three independent experiments. For details on cultivation and virus infection of the macrophages and the origin of the isolates see Fig. 1 and Table 1. The T cells were cultured in the presence of 10% exogenous IL-2 (Advanced Biotechnologies) according to a well-established technique (*30*). Autologous macrophages and T cells (about 2×10^6 per dilution) were exposed separately for 1 hour to tenfold (experiment 1) or serial (1:2, 1:5 and so on, experiments 2 and 3) dilutions of the virus inoculum. The virus was diluted in RPMI 1640 supplemented with 20% FBS and antibiotics. Treatment with Polybrene was omitted. The undiluted HTLV-III_B inoculum contained 5×10^5 to 10×10^5 cpm of RT activity per milliliter while all others contained 10^5 cpm of RT activity per milliliter. Susceptibility is expressed in relative values as the ratio of the end point dilution of the virus inoculum that was able to productively infect the macrophages compared to that able to infect the T cells.

Virus isolate	Macrophages/T cells		
	Experiment 1*	Experiment 2†	Experiment 3†‡
HTLV-III_B	0.0001	0.0005	0.0001
HTLV-III_{RC-br}	10.0	50.0	100.0
HTLV-III_{RC-PB}	1.0	1.0	10.0
HTLV-III_{Ba-L}	10.0	20.0	100.0

*Tenfold dilutions of virus preparations. †Serial dilutions of virus preparations. ‡T cells in this experiment were infected after having been maintained for 6 days in IL-2–containing medium.

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23. Undiluted culture fluids harvested from virus-infected cultures were used in the transmission studies. The undiluted virus inoculum (culture fluid) of HTLV-III_B and HTLV-III_{MN} contained 0.5×10^5 to 10×10^5 cpm of RT activity per milliliter, while all others contained 10^5 cpm/ml. Cells were treated with Polybrene (2 μg/ml) for 20 to 30 minutes, exposed to 1 ml of the virus inoculum for 1 hour at 37°C, and then washed with PBS and maintained in complete culture medium.

24. HTLV-III/LAV–infected and uninfected macrophages were detached from the surface of plastic flasks as follows. Macrophage layers were washed with PBS, Ca^{2+}-Mg^{2+}–free Hanks balanced salt solution was added, the flasks were incubated on ice for 15 to 30 minutes, and then the cells were gently scraped into the Hanks solution, washed with PBS, and processed for indirect immunofluorescence assay as described (*10*).
25. Virus production was detected in culture fluids by RT assay (*11*). RT activity was measured at 3- to 5-day intervals and expressed in counts per minute per milliliter of harvested culture fluid. Cell cultures were considered to be positive for virus infection only if an increase in RT activity was observed in culture fluids harvested from the same cultures during subsequent RT assays (see Fig. 4). In all cases analyzed, there were unambiguous correlations between positivity for RT activity in culture fluids and the percentage of cells positive for HTLV-III/LAV p17. Similarly, cultures exposed to the virus inoculum were considered negative for infection if no or only low and decreasing RT activity was observed during at least 4 weeks of cultivation in vitro.
26. Samples of high molecular weight DNA were prepared by standard methods (*27*). The samples were subjected to electrophoresis in 0.7% agarose gels, transferred to nitrocellulose (*28*), and hybridized as described (*14*). The probe used was either the Sst 1–Sst 1 region from the BH-10 clone (*29*) or a mixture of the 5′ and 3′ ends of the HXB-2 clone (*14*), each representing an 8.9-kb-long fragment of the HTLV-III_B genome.
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8 April 1986; accepted 6 June 1986

Transmission of a Female Sex Pheromone Thwarted by Males in the Spider *Linyphia litigiosa* (Linyphiidae)

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When a male Sierra dome spider (*Linyphia litigiosa*) encounters a virgin female that has been sexually mature for 7 to 10 days, he rapidly packs the silk of her web into a tight mass. This behavior hinders evaporation of a male-attractant chemical that such highly receptive females apply to their webs. The male thereby reduces the likelihood that his mating partner will attract rival males.

IN MANY ANIMAL SPECIES, MALES COMPETE intensely for sexually receptive females. Since aggressive interactions with rival males are likely to entail energetic expenditures and physical risks, males who are able to minimize such costs while still achieving matings will be at a selective advantage. One tactic males may use to reduce direct confrontations with rivals is to counteract sexually attractive signals being issued by females they locate.

Certain male beetles (*1*), moths (*2*), solitary bees (*3*), and garter snakes (*4*) produce an odor that renders females less attractive to other males. In these cases, male scent contributions probably signal rival males who detect the female that she has already mated or is about to copulate. Males would be deterred by such a signal only in species whose females become nonreceptive after copulation or are invulnerable to takeover attempts (*5*). In any other circumstance, the female's signal must be completely blocked. However, as far as I know, examples of animals truly muting communicative signals of conspecifics as a means of concealing sexual partners from potential usurpers, particularly cases involving clear costs to the blocked individual, are nonexistent. Here I report a novel behavioral mechanism used by male Sierra dome spiders (*Linyphia litigiosa*) to thwart transmission of a male-attractant pheromone facultatively produced by highly receptive virgin females.

The Sierra dome spider occurs throughout mountainous regions of western North America. I studied the reproductive behavior of this spider along Flathead Lake, Montana, from 1979 to 1985. Here the Sierra dome breeds from late June through early September. During this time, females are solitary and sedentary, constructing semipermanent dome-shaped webs used in prey capture and in which all sexual encounters occur.

Mature males are nomadic throughout the breeding season, wandering in search of webs of potential mates where they pause to guard and court the resident female. Intense combat for possession of females is common. In my dense study population (*6*), 96% ($n > 250$) of all females are guarded by a male at the time of their final molt, when they become sexually mature. These females inevitably copulate with the male currently guarding them. Although most females mate more than once, their sperm utilization pattern is one of strong first-male priority (*7*).

Table 1. Sections of the T-rod (Fig. 2A) from which males descended to the table during exposure to the three categories of females. Numbers in parentheses are expected frequencies.

Female in cage	Frequency of male descent		
	Near cage	Center	Far from cage
Virgin, intact web	22 (17.00)	7 (6.50)	4 (9.50)
Nonvirgin, intact web	2 (5.67)	3 (2.17)	6 (3.17)
Virgin, reduced web	10 (11.33)	3 (4.33)	9 (6.33)

Females were forced to remain unmated in the laboratory for 7 to 10 days after maturation; such an unmated condition might occur regularly in low-density populations. When these females were brought to the field and introduced onto webs from which mated females had been expelled (*8*), they elicited two intriguing male responses: (i) a greatly increased rate of male visitation compared to simultaneously monitored webs inhabited by mated females and (ii) a behavior by early-arriving males, termed web reduction, in which the male rapidly excised and gathered large portions of the dome of the female's web into a dense rope or ball (Fig. 1). Web reduction typically was completed within 15 to 30 minutes of the male's arrival upon the female's web and, without exception, was immediately followed by mating.

Introductions of mature virgin females onto individual webs in the field were performed 26 times. Mean male visitation rates were an order of magnitude greater for webs inhabited by these virgin females than for 184 simultaneously monitored webs occupied by mated females [0.29 ± 0.07 (SEM) and 0.03 ± 0.01 males per hour, respectively ($P < 0.01$)] (*9*). Sixty-nine percent of the 29 males arriving at the webs of mature virgin females performed web reduction, but none of the 63 males entering webs occupied by mated females displayed the behavior. Additionally, I monitored more than 625 natural intersexual pairings of Sierra dome spiders involving more than 200 previously mated females and never saw evidence of web reduction. Introductions of more than 175 mated females onto vacant webs never resulted in either an increased rate of male attraction or web-reduction behavior.

Researchers (*10, 11*) have described web reduction in natural populations of two other species of linyphiid spiders. They suggested that web reduction is triggered by a behavioral or pheromonal cue from highly

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