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Impaired Responsiveness to Gamma Interferon of Macrophages Infected with Lymphocytic Choriomeningitis Virus Clone 13: Susceptibility to Histoplasmosis

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Lymphocytic choriomeningitis virus clone 13 (LCMV clone 13), a variant isolated from the spleens of neonatally infected mice, causes persistent infections in mice infected as adults. Such persistently infected mice succumb to a normally sublethal dose of *Histoplasma capsulatum*, and their macrophages contain overwhelming numbers of yeast cells of the fungus. Both LCMV clone 13 and *H. capsulatum* yeast cells target and replicate in macrophages of the host. We sought to study the effects of LCMV clone 13 on the ability of macrophages to control growth of *H. capsulatum* in vitro. We show that the growth of *H. capsulatum* within macrophages was not directly affected by the presence of LCMV clone 13. However, macrophages containing LCMV clone 13 did not respond fully to gamma interferon (IFN-γ) stimulation. Such unresponsiveness resulted in proliferation of the fungus within macrophages cultured in the presence of IFN-γ. The addition of anti-IFN-α/β antibodies to LCMV clone 13-infected macrophage cultures restored macrophage responsiveness to IFN-γ. These results indicate that production of IFN-α/β by LCMV clone 13-infected macrophages antagonizes their responsiveness to IFN-γ. Such antagonism may be one of the mechanisms by means of which certain viruses cause immune suppression and susceptibility to opportunistic infections.

Lymphocytic choriomeningitis virus clone 13 (LCMV clone 13), a variant of LCMV, was isolated from the spleens of adult mice infected as neonates by the parental Armstrong strain virus (2). It is a macrophage-tropic variant and shows enhanced replication in macrophages in vivo and in vitro (17). Two amino acid changes in the viral polymerase and glycoprotein are responsible for the macrophage tropism (17, 18). This macrophage-tropic phenotype of clone 13 is the basis for its ability to establish a chronic infection (1, 3, 13, 18). Adult mice infected with LCMV clone 13 do not mount an effective immune response against *Histoplasma capsulatum* and succumb to a normally sublethal dose of the intracellular fungus (28). In contrast, mice infected with the parental Armstrong strain of LCMV survive the fungal challenge.

Macrophages play an essential role in natural as well as acquired immunity (12). Impairment of macrophage functions can severely compromise the immune system. It has been shown that viral infections in general impair macrophage functions (21). Although LCMV is a noncytolytic virus, infection with it impairs the functioning of these crucial cells of the immune system. Macrophages are the primary residence of *H. capsulatum* in an infected host (27), and disturbance of their functioning by a virus infection would alter their ability to deal with the fungus.

In this study, we examined the effect of LCMV clone 13 on the macrophage-fungus relationship in an in vitro system. The results indicate that clone 13 infection compromises macrophage responsiveness to gamma interferon (IFN-γ) and that such reduced responsiveness results in the uncontrolled proliferation of *H. capsulatum.*

MATERIALS AND METHODS

Animals. C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Seven- to nine-week-old mice were used in all experiments.

Virus. LCMV clone 13 originally isolated from the spleens of neonatally infected mice was propagated in Vero cells (2).

Fungus. Yeast cells of *H. capsulatum* 505 were grown on blood-cysteine-glucose agar slants at 37°C (26). Yeast cells from fresh 72-h slants were used in each experiment. A loopful of yeast cells was suspended in RPMI 1640, and the clumps were allowed to settle before the supernatants were collected for enumeration of yeast cells. The yeast cells remaining in the supernatants were mainly singlets and doublets.

Intracellular replication of *H. capsulatum* in macrophages. Peritoneal cells were harvested from normal mice as described previously (26). Macrophages were enriched by adherence to Formvar-coated (0.5% Formvar prepared in ethylene dichloride) coverslips (14, 26), and nonadherent cells were removed by washing the monolayer with warm medium. LCMV clone 13 was added to macrophage monolayers at a multiplicity of infection of 3. Infected macrophage monolayers and control uninfected monolayers were incubated at 37°C for 40 h. After incubation, *H. capsulatum* yeast cells were added to the monolayers with or without virus at a yeast-to-macrophage ratio of 1:1. After 2 h of phagocytosis, the monolayers were washed with medium to rid them of free yeast cells. Control coverslips were fixed at this time (T_0) in methanol. The rest of the coverslips were incubated for another 18 h before washing and fixation. Coverslips were stained with May Grunwald-Giemsa stain. The number of yeast cells in 100 to 150 macrophages per coverslip was determined. Growth was recorded as the difference in the mean numbers of yeast cells in macrophages between T_{18} and T_0.

In those experiments designed to assay for the effect of LCMV clone 13 on macrophage activation by IFN-γ, recombinant murine IFN-γ (rMuIFN-γ) was added to monolayers 4 h before the yeast cells (see protocol in preceding paragraph). Such interferon-exposed monolayers were incubated in media containing IFN-γ throughout the rest of the experiment. The rMuIFN-γ (lot no. M3-RD48) was supplied by Genentech, Inc. (South San Francisco, Calif.), and had a specific activity of 5.2×10^6 U/ml (endotoxin was <10 pg/ml).

In experiments designed to assess the potential roles of IFN-α/β in antagonism of IFN-γ immune stimulation, 200 U of rabbit anti-mouse IFN-α/β antibodies (Lee Biomolecular, San Diego, Calif.) per ml or normal rabbit serum was added to the monolayer 1 h after infection with virus.

Staining. Infection of macrophages by LCMV clone 13 was examined by use

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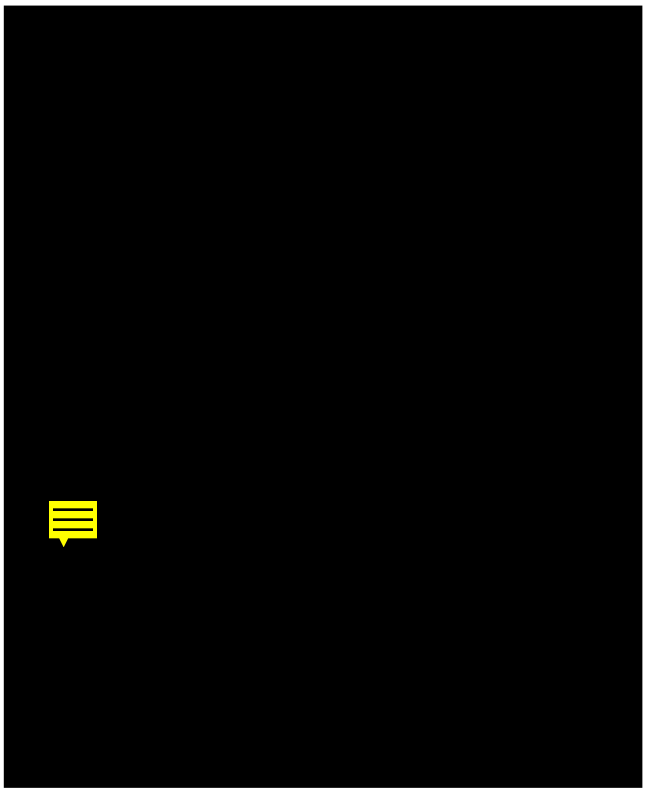

FIG. 1. Macrophages harbor both LCMV clone 13 and *H. capsulatum* yeasts. The presence of viral antigen and yeast nuclei within macrophages was detected by staining with guinea pig anti-LCMV antibody and FITC-conjugated rabbit anti-guinea pig antibody, with Hoechst reagent added to the latter. Note that Hoechst reagent stains nuclei of both macrophages and yeast cells. Green fluorescence shows cells infected with the virus (A), and blue fluorescence shows the cells which house the yeast cells (B). Open arrows point to macrophages infected with both agents and solid arrows point to macrophages infected with LCMV clone 13.

of polyclonal anti-LCMV guinea pig serum and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-guinea pig antibody (Cooper Biomedical, Inc., Malvern, Pa.). Hoechst reagent was used to stain the nuclei of macrophages and yeast cells. Coverslips were fixed in methanol; this was followed by fixation in acetone before the addition of anti-LCMV antibody. Hoechst reagent was added at the same time as FITC-conjugated antibody. Cells infected by either LCMV clone 13 or *H. capsulatum* yeast cells or by both were visualized under a fluorescent microscope with different filters. LCMV clone 13-infected macrophage monolayers stained with normal guinea pig serum and FITC-conjugated rabbit anti-guinea pig antibody were fluorescence negative, ruling out the possibility of nonspecific staining by guinea pig serum.

RESULTS

Macrophages as target cells for both LCMV clone 13 and *H. capsulatum*. The photographs in Fig. 1 show a macrophage monolayer stained by both anti-LCMV antibody and Hoechst reagent. Cells stained fluorescent green (Fig. 1A) were infected by LCMV, and those exhibiting tiny blue dots in the cytoplasm had phagocytosed the fungus (Fig. 1B). These micrographs show that the macrophages were targets for both LCMV clone 13 and *H. capsulatum*. However, the two infectious agents did not necessarily target the same macrophages. Some macrophages were infected with both agents (solid arrows), while others were infected with either LCMV clone 13 (open arrows) or *H. capsulatum* (data not shown).

Growth of *H. capsulatum* in macrophages is not affected by LCMV clone 13. Since both infectious agents targeted macrophages, it was important to determine whether replication of *H. capsulatum* in macrophages was directly affected by virus infection. At 40 h after virus infection, macrophages were allowed to ingest *H. capsulatum* yeast cells. Growth of the fungus in virus-infected macrophages was compared with that in normal macrophage monolayers. The results presented in Table 1 show that there was no difference in the levels of growth of *H. capsulatum* within macrophages infected or uninfected by LCMV clone 13.

TABLE 1. Growth of *H. capsulatum* in macrophages is not affected by LCMV clone 13 infection[a]

Macrophage infection by LCMV clone 13[b]	Time after phagocytosis (h)	Mean no. of yeasts/parasitized macrophage[c]	Growth[d]
−	0	2.5 ± 0.3	
−	18	7.9 ± 1.0	5.4
+	0	2.5 ± 0.4	
+	18	7.7 ± 1.2	5.2

[a] Resident macrophages were obtained from the peritoneal cavities of C57BL/6 mice.
[b] Macrophages were infected with LCMV clone 13 in vitro at a multiplicity of infection of 3, 40 h before *H. capsulatum* yeast cells were added to the culture for ingestion. −, uninfected macrophages; +, infected macrophages.
[c] Values are means and standard deviations from five separate experiments.
[d] Growth is calculated as the difference between the mean numbers of yeast cells per infected macrophage at 18 and 0 h after phagocytosis.

IFN-γ-induced antihistoplasma activity of macrophages is compromised by LCMV clone 13. Macrophages are not only the primary residence of *H. capsulatum* in an infected host but also the effector cells that eventually clear the infection (27). We have previously reported that murine peritoneal macrophages stimulated by IFN-γ acquire the ability to restrict the growth of *H. capsulatum* (26). However, macrophage monolayers infected by LCMV clone 13 did not respond to IFN-γ as the normal macrophages did (Table 2). Although stimulated by the same concentration of IFN-γ, the antihistoplasma activity of virus-infected macrophages was lower than that of normal macrophages (Table 2). Thus, infection with virus compromised the IFN-γ-induced macrophage antihistoplasma activity.

Soluble factor(s) released by LCMV clone 13-infected macrophages antagonizes activation by IFN-γ. By double-staining methods, we determined that approximately 70% of the macrophages in the monolayer stained positive for LCMV after 40

TABLE 2. Macrophage activation by IFN-γ is compromised by LCMV clone 13 infection[a]

Macrophage infection by LCMV clone 13[b]	IFN-γ stimulation[c]	Mean no. of yeasts/parasitized macrophage at T_{18}[d]	Growth[e]	% Inhibition of growth
−	−	7.9 ± 1.0	5.7	
−	+	2.3 ± 0.4	0.1	98
+	−	7.7 ± 1.2	5.5	
+	+	4.0 ± 0.7	1.8	67

[a] Resident macrophages were obtained from the peritoneal cavities of C57BL/6 mice.
[b] Macrophages were infected with LCMV clone 13 in vitro at a multiplicity of infection of 3, 40 h prior to IFN-γ stimulation. −, uninfected macrophages; +, infected macrophages.
[c] Macrophages were pretreated with rMuIFN-γ (2,000 U/ml) for 4 h before *H. capsulatum* yeast cells were added to the culture for ingestion. After 2 h of phagocytosis, IFN-γ was added back to the cultures. −, no IFN-γ stimulation; +, IFN-γ stimulation.
[d] Values are means and standard deviations from five separate experiments. Average T_0, 2.2 ± 0.4.
[e] Growth and percent inhibition of growth were calculated as explained in Materials and Methods.

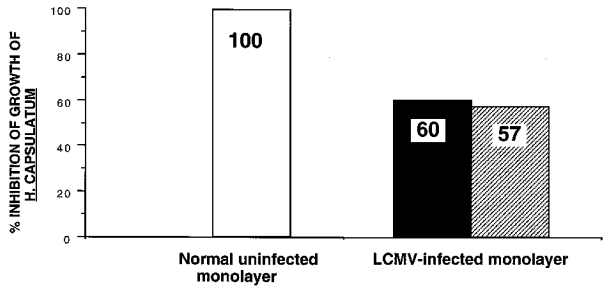

FIG. 2. The effector function of all macrophages in a monolayer infected by LCMV clone 13 is compromised regardless of the presence of virus. Normal (□) as well as LCMV clone 13-infected macrophage monolayers were treated with 2,000 U of rMuIFN-γ per ml. The numbers of yeast cells at T_{18} in macrophages infected (■; 5.90 ± 1.20) and uninfected (▨; 5.65 ± 1.45) by LCMV were scored separately. The numbers of yeast cells in macrophages infected with LCMV clone 13 were 2.75 ± 0.25 at T_0 and 10.00 ± 1.10 at T_{18}. Sixty percent of the macrophages in the LCMV-infected monolayer stained positive for LCMV. An average of 24.6% ± 1.3% of all macrophages in the LCMV clone 13-infected macrophage monolayer and 26.0% ± 1.0% of macrophages in the uninfected normal monolayer phagocytosed yeast cells. The percent inhibition of growth of *H. capsulatum* within IFN-γ-treated macrophages was calculated relative to the growth of the fungus within normal macrophages.

h of infection. Thus, not all macrophages in the monolayer exposed to LCMV clone 13 at a multiplicity of infection of 3 were infected by the virus. There were some macrophages that were not stained by anti-LCMV antibody but harbored the fungus. Thus, it was a question of whether macrophage infection by the virus compromised the antihistoplasma activity or, rather, soluble molecules released by infected macrophages affected all macrophages in the same monolayer regardless of the physical presence of the virus in the cells. To address this question, we compared the number of yeast cells in macrophages with virus and in those without virus, as determined by positive staining with anti-LCMV antibody. Data from these experiments are presented in Fig. 2. Macrophage antihistoplasma activity is presented as the percent inhibition of *H. capsulatum* growth. The growth of yeast cells in normal macrophages was completely inhibited (100%). While the antihistoplasma activity of virus-infected macrophages was compromised, whether or not the macrophages contained virus did not make a difference. Thus, we concluded that it was not the physical presence of virus which affected macrophage antihistoplasma activity but a soluble factor(s), released by virus-infected macrophages, that affected all cells in the same monolayer.

The sensitivity of the anti-LCMV immunofluorescence assay is limited, such that lightly infected cells will not stain with the antibody. Such cells would therefore appear in the wrong category in Fig. 2. However, our next set of experiments supports our conclusion.

Anti-IFNα/β antibodies reverse LCMV clone 13-induced macrophage unresponsiveness to IFN-γ. Virus infection induction of host cell production of soluble molecules has been documented (8). Among these molecules are IFN-α and IFN-β. Infection of macrophages by LCMV clone 13 induces low levels of IFN-α/β (16). The addition of neutralizing anti-IFN-α/β antibody (200 U/ml) to a macrophage monolayer infected by LCMV clone 13 increased the titer of infectious virus from $\log_{10}$ 4.7 to $\log_{10}$ 6.7. These data provided indirect evidence for the production of IFN-α/β by LCMV clone 13-infected macrophages.

On the basis of this observation, we studied whether or not production of IFN-α/β by macrophages infected by LCMV clone 13 affected their response to IFN-γ. Results from these experiments are presented in Fig. 3. Anti-IFN-α/β antibodies did not affect the growth of *H. capsulatum* in normal or LCMV-infected macrophages. Neither did the antibodies affect antihistoplasma activity induced by IFN-γ in normal macrophages. Neutralization of IFN-α/β in an LCMV clone 13-infected macrophage monolayer by the antibodies restored antihistoplasma activity. The IFN-γ induced *H. capsulatum*-inhibitory activity increased from 65.6% ± 7.8% to 92.2% ± 5.2%, a level that was comparable to that of normal macrophages. As a control, a separate experiment, using normal rabbit serum in place of rabbit anti-IFN-α/β antibody, was set up. In that experiment, IFN-γ induced 88.4% growth inhibi-

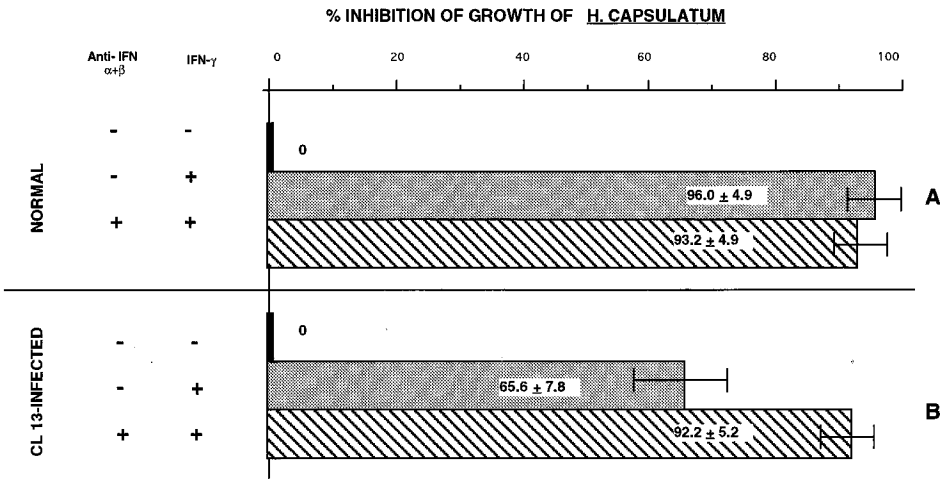

FIG. 3. Anti-IFN-α/β antibodies reverse LCMV clone 13-induced macrophage unresponsiveness to IFN-γ. Normal uninfected macrophages (A) and LCMV clone 13 (CL-13)-infected macrophages (B) were cultured in medium alone (■), in medium containing 2,000 U of rMuIFN-γ per ml (▨), or in medium containing both rMuIFN-γ (2,000 U/ml) and anti-IFN-α/β (200 U/ml) (▩). The values were calculated from data taken from three separate experiments. The mean numbers of yeast cells per *H. capsulatum*-infected macrophage at T_{18} are as follows: normal uninfected macrophages, 8.62 ± 0.29; normal uninfected macrophages with IFN-γ, 2.43 ± 0.30; normal uninfected macrophages with both anti-IFN-α/β and IFN-γ, 2.54 ± 0.07; LCMV clone 13-infected macrophages, 8.61 ± 0.61; LCMV clone 13-infected macrophages with IFN-γ, 5.17 ± 1.12; and LCMV clone 13-infected macrophages with both anti-IFN-α/β and IFN-γ, 2.64 ± 0.03. Treatment of macrophage monolayers with anti-IFN-α/β antibodies did not change their ability to phagocytose *H. capsulatum.*

tion. The addition of normal rabbit serum did not reduce IFN-γ-induced antihistoplasma activity (87.3%). Thus, it appears that infection with LCMV clone 13 antagonizes macrophage responsiveness to IFN-γ through the production of IFN-α/β.

DISCUSSION

In this study, we present data to show that macrophage effector function was compromised after infection by an immunosuppressive virus. The mechanism by which this virus compromised macrophage effector function involved induction of soluble IFN-α/β, which antagonized macrophage activation by IFN-γ.

IFN-γ is a key factor in the activation of macrophages for antihistoplasma activity. Mouse peritoneal and splenic macrophages stimulated by rMuIFN-γ acquire the ability to inhibit replication of the fungus (14, 26), and an animal's ability to produce IFN-γ coincides with its ability to clear the fungus (23). Therefore, production of IFN-γ and activation of macrophages by IFN-γ are both crucial to the ability of the infected animal to clear the fungus from its tissues.

Mice infected with LCMV clone 13 are susceptible to opportunistic fungal infections and die of a normally sublethal dose of *H. capsulatum* (28). Histopathological examination of tissue sections reveals large numbers of *H. capsulatum* yeast cells in tissue macrophages. This observation indicates that macrophages in animals infected by LCMV clone 13 do not acquire the ability to restrict replication of the intracellular fungi as do cells in normal animals (28). Yet production of IFN-γ by spleen cells in coinfected animals remains comparable to that in animals infected by the fungus alone (24). Thus, it appears that the inability of LCMV clone 13-infected animals to control replication of the fungus is not a result of a deficiency in IFN-γ production. We sought to study in vitro whether or not infection by LCMV clone 13 affected macrophage interaction with *H. capsulatum* and activation by IFN-γ. The results indicate that clone 13-infected macrophages allow growth of *H. capsulatum*, as do normal macrophages, and that virus interferes with macrophage activation by IFN-γ.

Infection of adult immunocompetent mice with LCMV clone 13 not only leads to a chronic infection but also results in suppressed T-cell immunity (1, 2, 28). Such mice have low levels of virus-specific cytotoxic T lymphocytes. The cytotoxic T-cell responses generated are not sufficient to clear the virus, but they are at least partially responsible for the immune-suppressed state (25). By employing macrophages in an in vitro system, we show that a macrophage-tropic virus causes immune suppression by induction of IFN-α/β. The IFN-α/β produced antagonizes macrophage activation by IFN-γ. The antagonistic effect of IFN-α/β is reversible by use of neutralizing antibodies.

It has been reported that type I interferons (IFN-α/β) antagonize IFN-γ-induced macrophage activation by interfering with expression of Ia antigens, regulation of mannosyl-fucosyl receptors, and binding of tumor necrosis factor alpha to receptors (5, 11, 15, 22). In addition, type I interferons also compromise macrophage effector functions such as H_2O_2 release, antitoxoplasma activity, and tumor cell killing (9, 19, 20). It is not clear, though, at what level these macrophage functions are blocked. It has been reported that IFN-α/β down-regulates the effect of IFN-γ on Ia expression by reducing steady-state levels of mRNA and gene transcription (6, 7). IFN-α/β-mediated antagonism of IFN-γ has also been attributed to the displacement of IFN-γ from its high-affinity receptors (29). As monocytes mature in vitro to become macrophages, both high- and low-affinity IFN-γ receptors appear on the surface (10). Low levels of IFN-α/β competitively block the binding of IFN-γ to its high-affinity receptors at a 100-fold-higher affinity than IFN-γ. This competitive displacement of IFN-γ by IFN-α/β results in reduced antitoxoplasma activity (29). It is possible that low levels of IFN-α/β produced by LCMV clone 13-infected macrophages result in competitive displacement of IFN-γ from its receptors, which then results in reduced antihistoplasma activity. However, before more work is done, this remains a speculation.

Recently, Diez et al. showed that the production of IFN-α/β and that of IFN-γ in animals are inversely correlated during acute toxoplasmosis (4). As the infection progresses, more IFN-α/β is generated and production of IFN-γ decreases. The authors suggested that IFN-α/β production is an important factor which is associated with lymphocyte mitogen unresponsiveness and reduced IFN-γ production. The systemic effect of IFN-α/β on lymphocyte functions in LCMV clone 13-infected animals and the susceptibility of animals to histoplasmosis still remain to be determined.

We show in this study that LCMV clone 13 infection of macrophages antagonizes the activation of macrophages by IFN-γ through the production of low levels of IFN-α/β. The mechanisms by which LCMV damages the immune system are complex and not fully understood. This finding offers an explanation for our in vivo observation that LCMV clone 13 infection of animals results in uncontrolled growth of *H. capsulatum* in macrophages. We offer the thesis that induction of IFN-α/β by virus is one possible mechanism by which virus infection induces immune suppression, manifested as an inability of the host to fight a secondary infection.

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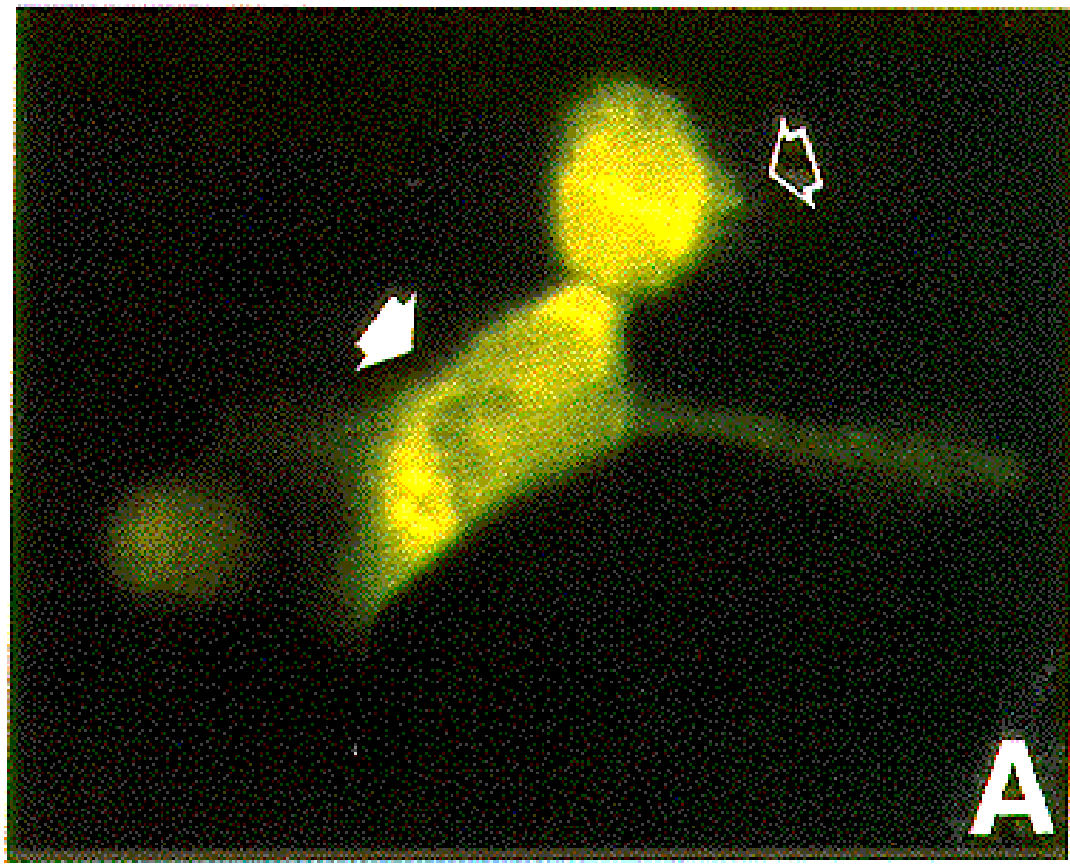
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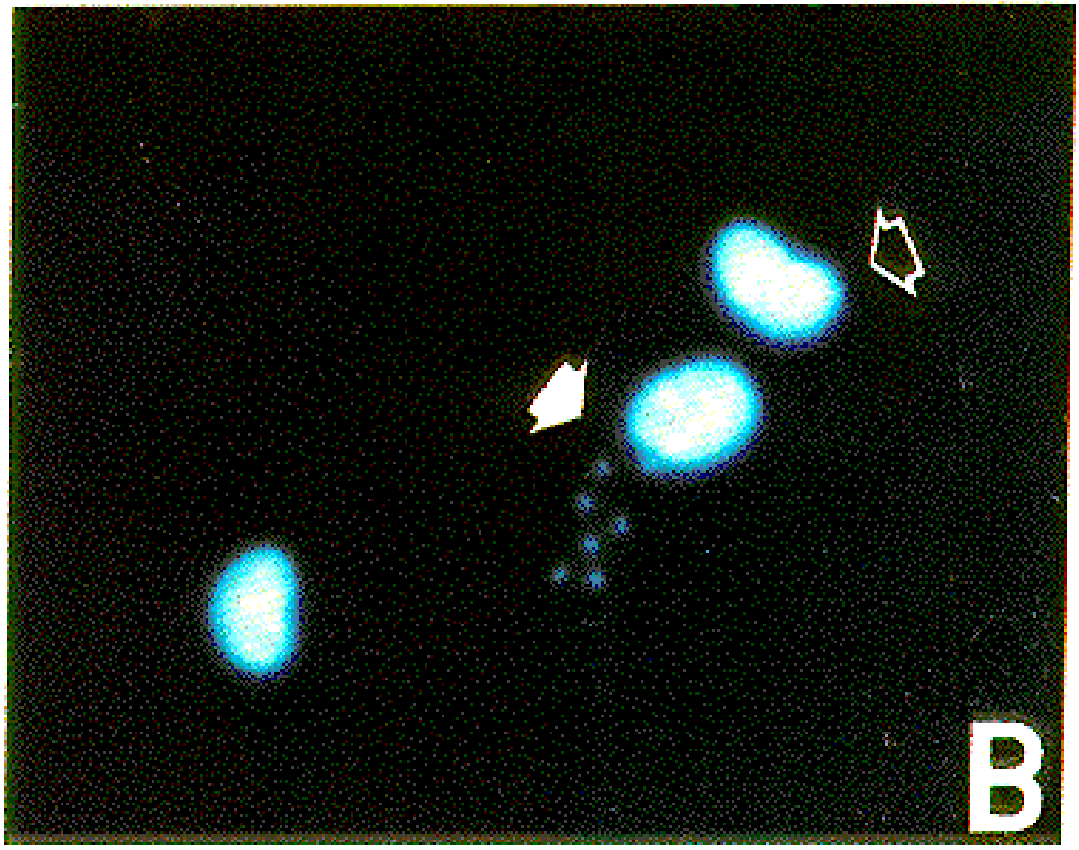
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