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Murine Natural Killer Cell Interactions with a Fungal Target, *Cryptococcus neoformans*

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Earlier investigations have shown that murine natural killer (NK) cells bind to and inhibit the growth of the fungal pathogen *Cryptococcus neoformans* in vitro and in vivo. To define the stages of NK cell-mediated inhibition of *C. neoformans* growth and the requirements for the completion of these stages, the events which lead to cryptococcal growth inhibition were compared with those previously elucidated for NK cell-mediated tumor cell lysis. Our data indicate that NK cell-cryptococci binding is a distinct event that precedes inhibition; is temperature independent, although it is slowed at 4°C; and is Mg^{2+} dependent. In contrast to binding, NK cell-mediated cryptococcal growth inhibition is temperature, Mg^{2+}, and Ca^{2+} dependent. The removal of Ca^{2+} by EDTA addition within 3 h after maximal NK cell-cryptococci binding significantly reduced cryptococcal growth inhibition, indicating that Ca^{2+} is required either late in the NK cell trigger stage or early in the inhibitory stage. These stages and requirements are similar to those previously demonstrated for the model of NK cell-mediated tumor cell lysis; however, the NK cell-cryptococci interactions are somewhat slower than the interactions which culminate in the lysis of the YAC-1 tumor cell targets. These results suggest that *C. neoformans* cells, although structurally distinct from the standard tumor cell targets, are capable of similar cell-to-cell interactions with NK effector cells as the tumor cell targets.

Intercellular communication, as a general biological phenomenon, has been an area of intensive study since the discovery, nearly 30 years ago, of pathways of direct communication between cells via the gap junction (10). Cell-to-cell interactions range from the actual cytoplasmic exchange of low-molecular-weight molecules, as seen in ionic and metabolic coupling, to highly specific receptor-ligand interactions which trigger a complex series of intracellular molecular events, as seen in the genetically restricted interactions between cells of the immune system (9, 12, 27). Regulation of the immune system through lymphocyte-lymphocyte and/or lymphocyte-accessory cell interactions has been the focus of numerous studies (15, 27, 44). Other recent studies have focused on the interactions of cytolytic effector cells, either cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells, with their potential target cells (8, 17, 26). In both the CTL and NK cell models of cell-mediated cytotoxicity against tumor cell targets, reports suggest that cell-to-cell communication is essential to the delivery of the lethal hit (2, 5, 6, 8, 23, 29, 30, 36, 41, 43). Specifically, in the model for NK cell-mediated lysis of tumor targets, distinct and seperate receptor-ligand interactions are required for NK cell-target cell binding and for NK cell triggering by the target cell for delivery of the lethal hit (30). Independent studies confirm that NK cell-tumor target binding alone is not sufficient to induce tumor cell lysis (1, 14, 30). For example, NK cells bind to, but do not lyse, gamma interferon-pretreated K562 tumor cell targets, which has been interpreted to suggest that the gamma interferon-treated K562 cells are no longer able to deliver the triggering signal to the NK cell that activates its lytic capabilities (7, 14). Also, Bonavida et al. (1) have suggested that NK cells isolated from patients with acquired immunodeficiency syndrome bind normally to tumor cell targets but lack the ability to be triggered by the target cell to deliver the lethal hit. In addition, others have demonstrated that the tumor target cell actively participates in the series of events which lead to its lysis (5, 6, 8, 46). Taken together, these reports indicate that cell-to-cell interactions play a major role in NK cell-mediated tumor cytotoxicity.

Previous studies in our laboratory have focused on NK cell interactions with a fungal target, *Cryptococcus neoformans*. *C. neoformans* is an encapsulated, yeastlike organism which causes human infection via the pulmonary route, ranging from an asymptomatic upper respiratory infection which is often spontaneously resolved by the host to a potentially fatal disseminated cryptococcosis which is generally manifest as meningitis. The low incidence of dissemination relative to the high frequency of exposure to this ubiquitous organism suggests a role for innate resistance mechanisms in the host defense against cryptococci. Our previous studies indicate that NK cells function in conjunction with other natural resistance mechanisms in the first-line host defense against cryptococci (18, 19). We have demonstrated that murine NK cells are able to bind to and inhibit the growth of *C. neoformans* both in vitro and in vivo (18, 19, 39, 40). However, *C. neoformans* is structurally distinct from the standard tissue or tumor cell target used in the model of NK cell-mediated cytotoxicity (38, 40). In contrast to the plasma membrane of the tumor cell, with which the NK cell directly interacts, the cryptococcal surface includes both a polysaccharide capsule and a cell wall with which the effector cell must first interact or penetrate before the cryptococcal cell membrane can be affected. In addition, although tumor cell lysis is the standard measure of NK cell-mediated tumor cytotoxicity, the cryptococcal cell wall makes lysis of cryptococci rare; therefore, inhibition of cryptococcal growth is used as an indication of NK cell-mediated damage to the cryptococcal cell. Considering the structural differences between the cryptococcal target and the standard tumor cell target, we have questioned whether the mechanisms of intercellular interactions in the model of

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NK cell-mediated cryptococcal growth inhibition are similar to those that have been elucidated for the model of NK cell-mediated tumor cell cytotoxicity.

Other investigators have resolved NK cell-mediated tumor cell cytotoxicity into several stages which are collectively termed the stimulus-secretion model. In simplified form, these stages include the following: (i) NK cell-target cell binding via a specific receptor-ligand interaction; (ii) activation of the lytic capabilities of the NK cell by the target cell via a second receptor-ligand interaction; (iii) delivery of the lethal hit, which culminates in target cell death; and (iv) killer cell-independent lysis of the target (22, 29). Our initial studies, which focused on the first stage of this model, i.e., NK cell-cryptococci binding, indicate that there are distinct physical and kinetic differences in NK cell-cryptococci target binding and growth inhibition in comparison with binding and lysis of the standard tumor cell target. Electron microscopy studies demonstrate that in contrast to the broad area of interdigitating membrane contact observed in NK cell-tumor cell conjugates and CTL-tumor cell conjugates, the contact area of NK cell-cryptococci conjugates is much smaller, does not appear to interdigitate, and is generally less intimate (20, 36a, 47). Further contrasting features of the NK cell-tumor cell model with the NK cell-cryptococci model are the times required for maximal binding and lysis or inhibition, which are 20 min for binding of NK cells to tumor cell targets followed by an additional 10 to 15 min to detect lysis and 2 h for maximal NK cell-cryptococci binding followed by an additional 6 h for measurable levels of growth inhibition to occur (36a, 42). Therefore, it seems likely that the structural differences between cryptococci targets and tumor cell targets do play a role in their physical interactions with NK cells.

The studies presented here were designed to delineate further the stages of NK cell-mediated cryptococcal growth inhibition and the requirements for the completion of each stage and to elucidate any differences in cell-to-cell interactions required to complete the inhibitory and lytic events by continually comparing the model of NK cell-mediated cryptococcal growth inhibition with that of NK cell-mediated tumor cell cytotoxicity through the use of parallel cryptococcal growth inhibition and ^{51}Cr-release assays.

MATERIALS AND METHODS

Mice. Female CBA/J mice (age, 5 weeks) were obtained from Jackson Laboratories, Bar Harbor, Maine. The animals were maintained in the University of Oklahoma animal facility until they were used for these studies at 7 to 8 weeks of age.

Reagents. EDTA, EGTA [ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], $MgCl_2$, and $CaCl_2$ were prepared in complete medium (CM) consisting of RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. All reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

Fungal target. *C. neoformans* 184A was maintained on modified Sabouraud agar slants (37). After 3 days of growth at room temperature, blastoconidia were harvested, washed three times in sterile physiological saline, and adjusted to the desired cell concentration for each experiment with CM. Cell concentrations were based on hemacytometer counts and were confirmed by determining the CFU on modified Sabouraud agar plates.

Effector cells. To enrich for NK cell activity, murine splenic nylon wool-nonadherent (NWN) cells were isolated as previously described, adjusted to the appropriate concentration for each assay with CM, and used as effector cells in these studies (18, 25).

***C. neoformans* growth inhibition assay.** To assess NK cell-mediated inhibition of *C. neoformans* growth, the assay previously described by Murphy and McDaniel (38) was used. Briefly, 1×10^6 splenic NWN effector cells and 2×10^3 cryptococcal target cells suspended in CM were added to quadruplicate wells of a flat-bottom, 96-well microtiter plate (Linbro Scientific Co., Hamden, Conn.). Quadruplicate control wells contained cryptococcal target cells in CM. Both experimental and control well volumes were adjusted to 0.25 ml with either CM or the variable reagent to be assayed appropriately diluted in CM. Cryptococcal controls containing the variable to be assayed were included to ascertain that the culture conditions alone were not inhibitory to cryptococcal growth. After incubating the plates for 18 h at 37°C in 5% CO_2, the contents of each well was serially diluted in sterile physiological saline, and 1 ml from each final dilution was plated in duplicate onto modified Sabouraud agar plates. After 3 days of incubation at room temperature, CFU were enumerated and the percentage of cryptococcal growth inhibition was determined by the following formula: percent cryptococcal growth inhibition = [(mean control CFU − mean experimental CFU)/mean control CFU] × 100.

YAC-1 cytolytic assay. To assess the level of splenic NK cell activity, we performed a standard assay measuring the ^{51}Cr released from YAC-1 target cells as previously described (18, 28, 38). YAC-1 cells were maintained in CM. Briefly, 10^6 splenic NWN effector cells suspended in CM were dispensed into quadruplicate wells of a round-bottom, 96-well microtiter plate (Linbro Scientific). After incubation for 1 h at 37°C in 5% CO_2 with 100 μCi of sodium chromate (Amersham Corp., Arlington Heights, Ill.), ^{51}Cr-labeled YAC-1 target cells were washed and suspended in CM, and 2×10^4 target cells were added to each well. The final volume of each well was adjusted to 0.2 ml with CM or the variable to be assayed appropriately diluted in CM. After incubating the plates for 4 h at 37°C in 5% CO_2 and centrifuging, 0.1 ml of supernatant was collected from each well and counted in a gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). For determinations of spontaneous release, 0.1 ml of supernatant from wells containing ^{51}Cr-labeled YAC-1 target cells and CM was counted; for determination of maximum release, a similar volume of supernatant from wells containing ^{51}Cr-labeled YAC-1 target cells and 2 N HCl was counted. In the experiments in which the effects of a variable reagent on YAC-1 cytolysis were assessed, appropriate spontaneous release and maximum release control wells containing an equivalent concentration of the variable as that used in the experimental wells were included. The percentage of ^{51}Cr released from YAC-1 target cells was calculated by using the following formula: percent ^{51}Cr released = [(counts per minute of experimental supernatant − counts per minute of spontaneous release control)/counts per minute of maximum release control] × 100. Numerous studies have demonstrated that the percentage of ^{51}Cr released is a direct correlate of the percentage of cytotoxicity or the percentage of lysis of YAC-1 targets (28, 31). Obtaining the expected results, based on previous reports (21, 22, 35, 41, 42), in the ^{51}Cr-release assays, which were performed in parallel with the cryptococcal growth inhibition assays in these studies, allowed us to ascertain

that the various culture conditions did not adversely effect the activity of the effector cells.

Assay for effector cell-target cell conjugate formation. Splenic NWN effector cells were incubated with YAC-1 target cells or *C. neoformans* target cells in polystyrene tubes (12 by 75 mm; Falcon; Becton Dickinson Labware, Oxnard, Calif.) at an effector cell to target cell ratio of 2:1 in 0.2 ml of CM or a designated reagent diluted in CM. After incubating effector cells with cryptococcal target cells for 2 h at 37°C in 5% CO_2, a time previously determined to be optimal for maximal NK cell-cryptococci conjugate formation (36a), the conjugate suspension was stained with 0.25% alcian blue in serum-free RPMI 1640 medium (pH 6.4), and a sample was counted by light microscopy. This technique facilitates differentiation of NK cells from cryptococci. Alcian blue binds to the polysaccharide capsule of the cryptococci, staining the cryptococci blue, whereas it does not stain the effector cells.

After incubating effector cells with YAC-1 target cells for 1 h at 37°C in 5% CO_2, a time previously determined to be optimal for maximal tumor target conjugate formation, a sample of the conjugate suspension was counted by light microscopy. The percentage of effector cells bound to target cells were calculated by counting a minimum of 200 effector cells.

Variable temperature assays. To assess the effects of temperature on splenic NWN effector cell-target cell conjugate formation, cryptococcal growth inhibition, and YAC-1 cytotoxicity, the three standard assays described above were performed at various incubation temperatures. First, the effector and target cells were incubated at 4, 25, or 37°C for the entire length of the incubation period of each of the three assays. Second, at designated times during a 4-h incubation period, binding of NWN effector cells to cryptococci targets was enumerated by using samples taken from tubes incubated under each condition, i.e., 4, 25, or 37°C. Finally, to determine whether shifting the samples from 4 or 25°C to an incubation temperature of 37°C would have an effect on the inhibitory or lytic event, the cryptococcal growth inhibition assay and the ^{51}Cr-release assay were performed with an initial incubation period of various lengths at 4 or 25°C, and then each assay was subsequently shifted to 37°C for a standard-length incubation period, i.e., 18 h for the growth inhibition assay or 4 h for the ^{51}Cr-release assay.

Determination of the requirements for Mg^{2+} and Ca^{2+}. To determine the requirements for Mg^{2+} and Ca^{2+} in effector cell-target cell conjugate formation, splenic NWN effector cells were incubated with cryptococci target cells for 2 h or with YAC-1 target cells for 1 h under Mg^{2+}- and/or Ca^{2+}-free conditions as well as Mg^{2+}- and/or Ca^{2+}-containing conditions, and the percent conjugate formation was determined as described above. The following culture conditions were assessed: (i) RPMI 1640 plus 10% FCS, a Mg^{2+}- and Ca^{2+}-containing condition; (ii) 2.5 mM EGTA plus 2.5 mM $MgCl_2$ (EGTA-Mg^{2+}), a Mg^{2+}-containing, Ca^{2+}-free condition; (iii) EGTA-Mg^{2+} plus 5.0 mM $CaCl_2$ (EGTA-Mg^{2+}-Ca^{2+}), a Mg^{2+}- and Ca^{2+}-containing condition; (iv) 10 mM EDTA, a Mg^{2+}- and Ca^{2+}-free condition; and (v) 5 mM EDTA plus 5.0 mM $CaCl_2$ (EDTA-Ca^{2+}), a Mg^{2+}-free, Ca^{2+}-containing condition. In addition, a 10-h rather than an 18-h cryptococcal growth inhibition assay and a standard 4-h ^{51}Cr-release assay were performed under similar culture conditions. A 10-h cryptococcal growth inhibition assay, in contrast to the 18-h assay, was used to minimize the cytotoxic effects of the culture conditions in the cryptococcal control wells. In both the cryptococcal growth inhibition assay and the ^{51}Cr-release assay, $MgCl_2$, EGTA, or EDTA was added at the onset of the incubation period, whereas $CaCl_2$ was added 2 h after the onset of incubation to the cryptococcal growth inhibition assay and 20 min after the onset of incubation to the YAC-1 cytolytic assay. Trypan blue viability staining after incubation periods of 1, 2, and 10 h ascertained that the culture conditions had no effect on NWN effector cell viability.

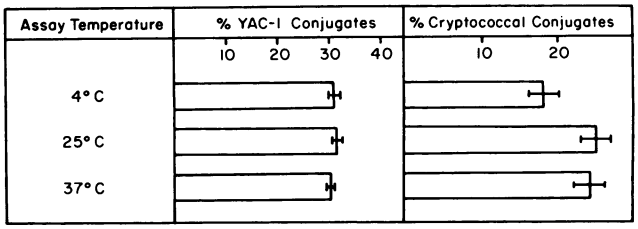

FIG. 1. Percent effector cell-target cell conjugates at incubation temperatures of 4, 25, and 37°C. Bars represent the mean ± standard error of the mean of triplicate counts of a minimum of 200 effector cells per count. Data are representative of three experiments.

Ca^{2+}-pulse technique. To determine the length of time that Ca^{2+} is required for NK cell lytic or inhibitory activity to occur after effector cell-target cell binding, we used a Ca^{2+}-pulse technique modified from the procedure of Hiserodt et al. (22). For the cryptococcal growth inhibition assay and the YAC-1 cytotoxicity assay, NWN effector cells were incubated with target cells at 37°C (*C. neoformans* for 2 h or YAC-1 for 20 min) in the presence of EGTA-Mg^{2+} to allow effector cell-target cell binding. After binding, $CaCl_2$ was added to a final concentration of 5.0 mM to initiate the Ca^{2+} pulse. At various times after the Ca^{2+} pulse, 10 mM EDTA was added to chelate all divalent cations, and then inhibitory or cytolytic activity was measured as described above at the end of the standard-length incubation period. EDTA was added at the following times after the Ca^{2+} pulse: 0.5, 1, 2, 3, 4, and 5 h for the cryptococcal growth inhibition assay and 5, 30, 60, and 90 min for the YAC-1 cytolytic assay.

Statistical analysis. Means, standard errors of the means, and two-tailed Student's *t* tests were used to analyze the data.

RESULTS

Effects of temperature on effector cell-target cell conjugate formation. The effects of incubation temperature on splenic NWN effector cell-target cell binding were assessed by incubating the effector cells with YAC-1 target cells or *C. neoformans* target cells at 4, 25, or 37°C. As previously reported (42), at each of the three incubation temperatures, NK cell–YAC-1 conjugate formation reached maximal levels and was equivalent at all temperatures after 1 h of incubation (Fig. 1). In contrast, NK cell-cryptococci conjugate formation was significantly ($P < 0.001$) less when the effector and target cells were incubated for 2 h at 4°C than when they were incubated for a similar time at 25 or 37°C (Fig. 1). These data suggest that NK cell-cryptococci conjugate formation is reduced at 4°C in comparison with that at 25 or 37°C. To determine the length of time required for NK cell-cryptococci conjugate formation to reach maximal levels at 4°C, we assessed conjugate formation at 0.5-h intervals through a 4-h incubation at each of the three incubation temperatures (Fig. 2). NK cell-cryptococci conjugate formation was retarded at

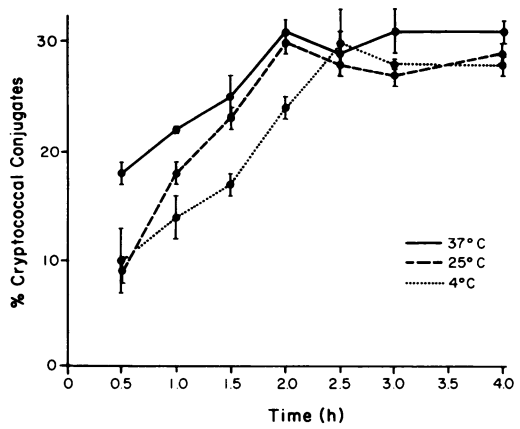

FIG. 2. Percent NK cell-*C. neoformans* conjugates at 0.5-h intervals through 4 h with assay temperatures of 4, 25, or 37°C. Points represent the mean ± the standard error of the mean of triplicate counts of 200 effector cells per count. Data are representative of three experiments.

4°C, requiring 2.5 h to reach levels comparable to those reached after 2 h of incubation at 25 or 37°C (Fig. 2).

Effects of temperature on effector cell-mediated lytic and growth-inhibitory activities. To assess the effects of temperature on splenic NWN cell-mediated YAC-1 cytolysis and cryptococcal growth inhibition, effector cells were incubated with ^{51}Cr-labeled YAC-1 target cells or viable *C. neoformans* blastoconidia for 4 or 18 h, respectively, at each of the three temperatures, i.e., 4, 25, or 37°C. Appropriate YAC-1 and cryptococci control groups were included at each of the three temperatures. As previously reported by other investigators (41, 42), the percent ^{51}Cr released from YAC-1 targets was minimal when the assay was incubated at 4 or 25°C compared with the lytic activity seen when the assay was incubated at 37°C ($P < 0.001$) (Fig. 3). Similarly, the percent cryptococcal growth inhibition was significantly reduced when the assay was incubated at 4 or 25°C compared with the results seen at 37°C ($P < 0.001$). Since effector cell-cryptococci binding occurred at 4 and 25°C, whereas cryptococcal growth inhibition was minimal at these reduced temperatures, we questioned whether shifting the samples to 37°C after maximal binding at the lower temperatures would allow the inhibitory event to proceed. Both the ^{51}Cr-release assay and the cryptococcal growth inhibition assay were incubated at 4 or 25°C for various times before the assays were shifted to 37°C for their respective standard-length incubation periods. When the ^{51}Cr-release assay was incubated at 4 or 25°C for 30 min, followed by shifting the assay to 37°C for an additional 4 h, the percent ^{51}Cr released from YAC-1 target cells was comparable to that seen when the assay was incubated for an equivalent total length of time at 37°C (21, 35) (Fig. 4). If the assay was incubated for longer periods, such as 1 or 2 h at 4 or 25°C and then shifted to 37°C, the level of NK cell activity was partially recovered but not fully comparable to that seen with an equivalent total length of incubation at 37°C. In contrast, the percent cryptococcal growth inhibition was comparable to that seen after the assay was incubated an equivalent total length of time (22 h) at 37°C only in the samples incubated for 4 h at 4°C followed by a shift to 37°C for 18 h (Fig. 4). The percent cryptococcal growth inhibition was not generally recovered by shifting the assay from an incubation temperature of 4 or 25°C to an incubation temperature of 37°C. In addition, the mean number of CFU in control samples containing cryptococci alone with media were not significantly different in samples incubated for 4 h at 4°C followed by 18 h at 37°C ($1.6 \times 10^5 \pm 0.1 \times 10^5$) from samples only incubated for 18 h at 37°C ($1.5 \times 10^5 \pm 0.2 \times 10^5$). Since the doubling time of the cryptococcal cells has been determined to be approximately 4 h at 37°C, these data indicate that in the samples incubated for 4 h at 4°C followed by 18 h at 37°C, replication of cryptococci was stopped at 4°C but returned to normal levels when the samples were shifted to 37°C, resulting in numbers of cryptococci equivalent to those in the samples which were only incubated for 18 h at 37°C.

Divalent cation requirements for effector cell-target cell conjugate formation, lytic activity, and inhibitory activity. The requirements for Mg^{2+} and Ca^{2+} in effector cell-cryptococci binding were determined by adding chelating agents to the 2-h conjugate formation assay. The results were compared with results of assays in which there was Mg^{2+} and/or Ca^{2+} present in the medium. Maximal effector cell-cryptococci binding occurred only in the Mg^{2+}-containing culture conditions, i.e., RPMI 1640 medium plus 10% FCS, EGTA-Mg^{2+}, and EGTA-Mg^{2+}-Ca^{2+}, whereas Ca^{2+} was not required for binding to occur, as shown by comparable binding in the Mg^{2+}-containing and Ca^{2+}-free culture condition (EGTA-Mg^{2+}) as that seen in the Mg^{2+}- and Ca^{2+}-containing samples (RPMI 1640 medium plus 10% FCS and EGTA-Mg^{2+}-Ca^{2+}) and by the absence of binding in the Mg^{2+}-free and Ca^{2+}-containing samples (EDTA-Ca^{2+}) (Fig. 5). Similarly, Mg^{2+} but not Ca^{2+} was required for effector cells to bind to YAC-1 target cells, as has been previously reported (22, 29) (Fig. 5).

To assess the requirements for Mg^{2+} and Ca^{2+} in NK cell-mediated cryptococcal growth inhibition, Mg^{2+}- and/or Ca^{2+}-free conditions as well as Mg^{2+}- and/or Ca^{2+}-containing conditions were imposed upon the cryptococcal growth inhibition assay by using chelating agents. Maximal

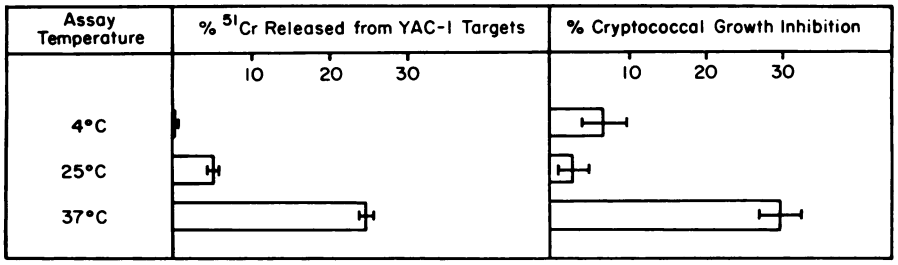

FIG. 3. Splenic NK cell activity in a 4-h ^{51}Cr-release assay and an 18-h cryptococcal growth inhibition assay at incubation temperatures of 4, 25, and 37°C. Bars represent the mean ± standard error of the mean of quadruplicate samples. Data are representative of three experiments.

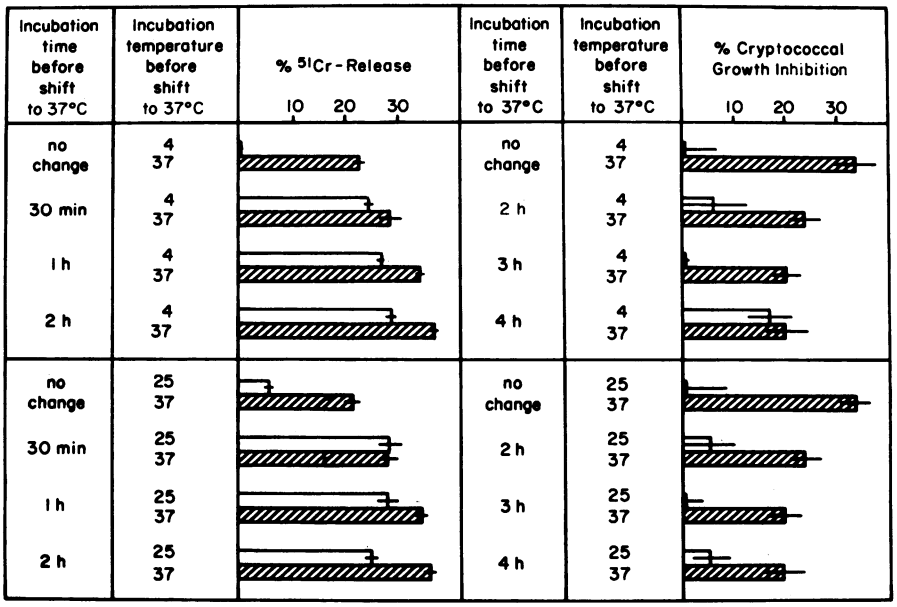

FIG. 4. Splenic NK cell activity in the ^{51}Cr-release assay and cryptococcal growth inhibition assay after initial incubations of effector cells and target cells for various lengths of time at reduced temperatures of 4 or 25°C followed by a shift of the samples to 37°C. Bars represent the mean ± standard error of the mean of quadruplicate samples. Data are representative of three experiments.

cryptococcal growth inhibition was only observed in the culture conditions in which both Mg^{2+} and Ca^{2+} were present, i.e., RPMI 1640 medium plus 10% FCS and EGTA-Mg^{2+}-Ca^{2+} (Fig. 6). Although maximal binding was achieved in the presence of Mg^{2+} without Ca^{2+} (Fig. 5), the inhibitory event did not proceed in the absence of Ca^{2+}, as shown by the lack of cryptococcal growth inhibition in the Ca^{2+}-free conditions, i.e., EGTA-Mg^{2+} and EDTA. However, Ca^{2+} alone without Mg^{2+} did not support cryptococcal growth inhibition, as shown by the lack of inhibitory activity in the EDTA-Ca^{2+}-treated samples. Similar requirements for Mg^{2+} and Ca^{2+} in NK cell-mediated YAC-1 lysis, as previously reported (22), were obtained by imposing the Ca^{2+}- and/or Mg^{2+}-free conditions as well as the Ca^{2+}- and/or Mg^{2+}-containing conditions upon the ^{51}Cr-release assay (Fig. 6).

The length of time Ca^{2+} must be present after binding for NK cells to mediate cryptococcal growth inhibition. To determine the length of the postbinding period in which Ca^{2+} is required for NK cell-mediated cryptococcal growth inhibition, we used a Ca^{2+}-pulse technique modified from the procedure of Hiserodt et al. (22). After maximal effector cell-cryptococci binding in EGTA-Mg^{2+}, Ca^{2+} was added to the assay to initiate the inhibitory event, followed by the addition of EDTA at 1-h intervals through 5 h to chelate all available divalent cations. When Ca^{2+} was removed from the assay by the addition of EDTA at times before 3 h after maximal effector cell-cryptococci binding, the percent cryptococcal growth inhibition was significantly reduced (Fig. 7). The addition of EDTA later than 3 h after the Ca^{2+} pulse had no significant effect on the percent cryptococcal growth inhibition. Results from the ^{51}Cr-release assay performed in parallel with the cryptococcal growth inhibition assay were similar to those previously reported by others (22). YAC-1 cytolysis was significantly reduced by the addition of EDTA up to 60 min after maximal NK cell–YAC-1 binding, and the addition of EDTA later than 60 min after the Ca^{2+} pulse had no effect on YAC-1 cytolysis (Fig. 7).

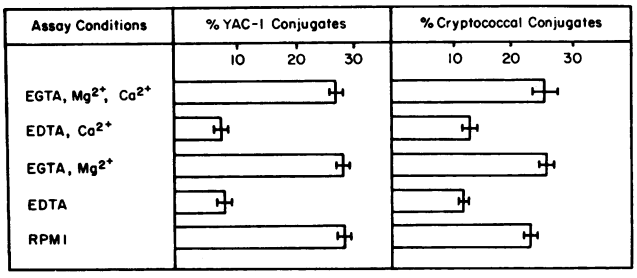

FIG. 5. Percent effector cell-target cell conjugates with Mg^{2+}- and/or Ca^{2+}-free culture conditions in comparison with Mg^{2+}- and/or Ca^{2+}-containing culture conditions. Bars represent the mean ± standard error of the mean of triplicate counts of at least 200 effector cells per count. Data are representative of three experiments.

DISCUSSION

Numerous studies of the interactions of cytolytic effector cells with tumor cell targets indicate that cell-to-cell communication is essential to completion of the lytic process (2, 5, 6, 8, 23, 29, 30, 41, 45). An ordered sequence of events has been defined in the process that leads to NK cell-mediated tumor cell lysis which demonstrates specific instances of intercellular communication between the effector and target cells (45). Having previously shown that NK cells bind to and inhibit the growth of the fungus, *C. neoformans*, and that this inhibitory activity correlates with tumoricidal activity, we designed these studies to begin to delineate the sequence of events which leads to NK cell-mediated cryptococcal growth inhibition and to compare these events with the ordered sequence of events previously elucidated for NK cell-mediated tumor cell lysis (22, 38, 39). Furthermore, defining the growth-inhibitory process allows us to compare the effector cell-target cell interactions which contribute to the inhibitory and lytic events and to determine whether a distinctly different target cell, such as a fungus, interacts

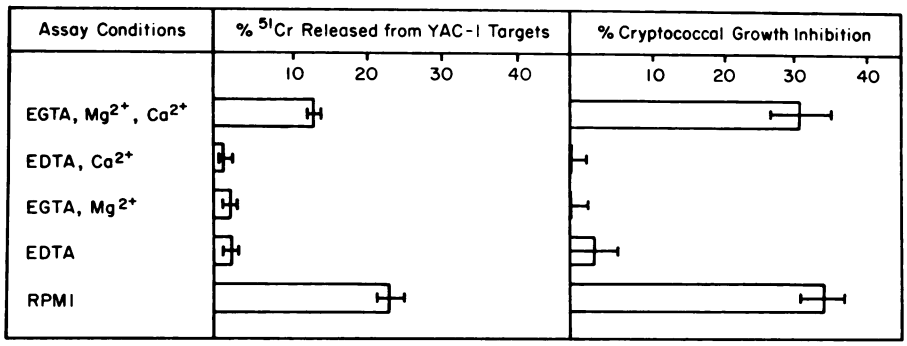

FIG. 6. Splenic NK cell activity in the 4-h ^{51}Cr-release assay and 10-h cryptococcal growth inhibition assay with Mg^{2+}- and/or Ca^{2+}-free culture conditions in comparison with Mg^{2+}- and/or Ca^{2+}-containing culture conditions. Bars represent the mean ± standard error of the mean of quadruplicate samples. Data are representative of three experiments.

with the NK effector cell in the same manner as the standard tissue cell-type target.

Previous investigators have determined that NK cell-tumor cell conjugate formation is a discrete step which precedes the lethal hit in NK cell-mediated tumor cytotoxicity (16, 45). Similarly, initial studies in our laboratory indicate that NK cell-cryptococci conjugate formation precedes NK cell-mediated cryptococcal growth inhibition (39). To assess the requirements for NK cell-cryptococci conjugate formation, we assayed NK cell-target cell binding while varying the assay temperature or the availability of divalent cations. The results indicate that NK cell-cryptococci binding is for the most part temperature independent, although binding is retarded at 4°C in comparison with that at 25 and 37°C (Fig. 1 and 2). In accordance with previous reports, NK cell–YAC-1 conjugate formation was also temperature independent, demonstrating equivalent binding at 4, 25, and 37°C (Fig. 1) (42). In contrast, other investigators have shown that CTL-target cell binding is temperature dependent and, therefore, is abrogated at 4°C (23, 32). Furthermore, our data indicate that NK cell-cryptococci binding, like NK cell–YAC-1 conjugate formation, is Mg^{2+} dependent and Ca^{2+} independent (Fig. 5). Mg^{2+}-dependent binding is also a characteristic of effector cell-target cell interactions in the CTL model of cell-mediated cytotoxicity (22, 23, 33–35). Taken together, our data indicate that the requirements for NK cell-cryptococci conjugate formation are, in general, similar to those previously elucidated in the NK cell-tumor cell target model, in that binding is Mg^{2+} dependent and Ca^{2+} independent. In addition, NK cell-cryptococci binding is temperature independent, but in contrast to NK cell–YAC-1 binding, it is significantly slowed at 4°C. Thus, there are some subtle differences in the types of cell-to-cell interactions that may be occurring in the process of NK cell-cryptococci conjugate formation in comparison with NK cell-tumor cell conjugate formation. It is possible that some of these differences could be related to the structural differences between the two types of target cells.

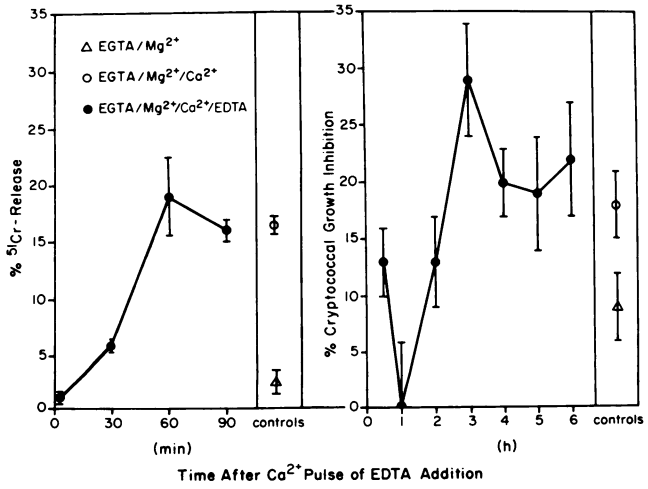

FIG. 7. Splenic NK cell activity in the 4-h ^{51}Cr-release assay and 10-h cryptococcal growth inhibition assay by using a Ca^{2+}-pulse technique. Each point represents the mean ± standard error of the mean of quadruplicate samples. Data are representative of two experiments.

In addition to defining the requirements for NK cell-*C. neoformans* conjugate formation, experiments were performed to begin to elucidate the postbinding events which culminate in cryptococcal growth inhibition. We assessed the effects of varying the assay temperature or the divalent cation availability on NK cell-mediated inhibition of cryptococcal growth. Our data indicate that the cryptococcal inhibitory event, similar to the tumor cell lytic event, is temperature, Mg^{2+}, and Ca^{2+} dependent. Cryptococcal growth inhibition and, as previously reported by others (42), YAC-1 cytolysis are completely abrogated at reduced temperatures. Both cryptococcal growth inhibition and ^{51}Cr release were minimal when the assays were incubated at 4 or 25°C, in comparison with that at 37°C (Fig. 3). However, when NK cell–YAC-1 binding was allowed to occur at 4 or 25°C and the assay was then shifted to 37°C, YAC-1 cytolysis was completely or partially recovered in comparison with that in the samples incubated at 37°C, depending upon the length of time that the assay was incubated at reduced temperatures (Fig. 4). In contrast, the growth-inhibitory activity was not generally recovered by shifting the cryptococcal growth inhibition assay to 37°C after binding at either of the reduced temperatures. Only in the samples incubated for 4 h at 4°C followed by 18 h at 37°C was the percent cryptococcal growth inhibition comparable to that seen after 22 h at 37°C (Fig. 4).

Several processes involved in general cell-to-cell interactions that have been shown to play a role in NK cell-mediated tumor cell lysis may also participate in the cryptococcal growth-inhibitory event. Membrane fluidity, receptor-mediated endocytosis, and enzyme activity are a few of the processes that function during NK cell-mediated tumor cell lysis that are adversely affected by reduced temperatures

(3–6, 8, 11, 24, 29, 30). However, the effects of reduced temperature on each of these mechanisms would be expected to be reversible upon shifting the assay temperature to 37°C. In accordance with this, as previously reported (22), NK cell-mediated YAC-1 cytotoxicity was mostly recovered after allowing binding at reduced temperatures followed by shifting the assay to 37°C (Fig. 5). In contrast, the negative effects of reduced assay temperature on NK cell-mediated cryptococcal growth inhibition were not easily reversed by shifting the assay from 4 or 25°C to the higher temperature of 37°C (Fig. 5). Since the same effector cells were used in parallel assays, these data suggest possible differences in the NK cell-cryptococci interactions which lead to inhibition of cryptococcal growth in comparison with the effector cell-target cell interactions which result in NK cell-mediated tumor cell cytotoxicity. The reduced assay temperature had an unknown effect on the role of the fungal target in the inhibitory assay that was not observed when tumor cells were the targets at similar reduced assay temperatures. However, it appears that the cryptococci were able to overcome these undefined effects and interact as usual in the samples that were incubated for 4 h at 4°C before being shifted to 37°C for 18 h, because cryptococcal growth inhibition was recovered in these samples and was comparable to that seen in those samples incubated at 37°C for 22 h.

Similar to the model of NK cell-mediated YAC-1 tumor cell cytotoxicity, cryptococcal growth inhibition was Mg^{2+} and Ca^{2+} dependent (Fig. 6), although neither Mg^{2+} nor Ca^{2+} alone could support NK cell-mediated cryptococcal growth inhibition. These data confirm that Mg^{2+}-dependent cryptococcal target cell binding is a discrete step which precedes a Ca^{2+}-dependent delivery of the inhibitory event, a situation similar to that in the model of NK cell-mediated tumor cell cytotoxicity. Furthermore, data from the Ca^{2+}-pulse assay indicate that Ca^{2+} is required for only 3 h postbinding, after which removal of available Ca^{2+} through chelating agents did not abrogate the growth-inhibitory process (Fig. 7). These data suggest that the Ca^{2+}-dependent stage of cryptococcal growth inhibition is an early postbinding event which is followed by a Ca^{2+}-independent stage(s) culminating in cryptococcal growth inhibition. Similar requirements for Mg^{2+} and Ca^{2+} were observed, as previously reported (20, 42), in the NK cell–YAC-1 model of tumor cell cytotoxicity (Fig. 7). Although the kinetics of the Ca^{2+} requirement in the NK cell–YAC-1 model were characteristically quicker than those of cryptococcal growth inhibition, with Ca^{2+} being required for only 60 min postbinding in comparison with 3 h in the NK cell-cryptococci model. These kinetics were expected when we considered that our previous data indicate that YAC-1 lysis can be detected 20 min after maximal NK cell binding, whereas cryptococcal growth inhibition is not detected until 6 h after maximal binding (20, 42).

In conclusion, our results indicate that NK cell-cryptococci conjugate formation precedes cryptococcal growth inhibition and is temperature independent, providing that the effector cells and target cells are incubated together for a sufficient time at reduced temperatures, and is Mg^{2+} dependent, whereas the inhibition of cryptococcal growth is temperature, Mg^{2+}, and Ca^{2+} dependent. The Ca^{2+}-dependent stage of the inhibitory event occurs within 3 h of maximal NK cell binding to the cryptococci and is followed by a Ca^{2+}-independent stage(s) which culminates in cryptococcal growth inhibition. Therefore, the initial stages of cryptococcal growth inhibition and the requirements for the completion of these stages are similar to those previously elucidated for NK cell-mediated tumor cell lysis, although the kinetics for completion of these stages are somewhat slower in NK cell-mediated cryptococcal growth inhibition than in NK cell-mediated tumor cell lysis. Additionally, the data suggest both similarities and differences in the types of cell-to-cell interactions which possibly participate in NK cell-mediated cryptococcal growth inhibition in comparison with NK cell-mediated tumor cell lysis. Therefore, it appears that, although structurally distinct from the usual tumor cell target, *C. neoformans* is capable of similar cell-to-cell interactions with the NK effector cell.

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