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Evidence for Involvement of β-Glucan-Binding Cell Surface Lectins in Human Natural Killer Cell Function

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We have studied the effects of yeast cell wall derivatives (zymosan and particulate β-glucan), on the cytolytic effector function of human natural killer cells. Both zymosan and particulate β-glucan were found to inhibit the NK-cell-mediated killing of K562, Molt-4, U937, and HL60 tumor cells. Zymosan also inhibited the IL-2-dependent proliferation of NK cells, suggesting that some component of the yeast cell wall delivers a down-modulatory signal affecting multiple NK cell functions. NK cell surface molecules capable of binding both zymosan and Sepharose-immobilized pustulan (linear 1,6-β-D-glucan, a carbohydrate component of zymosan and particulate β-glucan) were identified in detergent lysates prepared from surface iodinated NK cells. Our results suggest that NK cells express cell surface β-glucan-binding lectins that may contribute to NK-cell-mediated natural cytotoxicity.

INTRODUCTION

The vertebrate immune system has evolved a number of strategies for the specific recognition of invading pathogens. Whereas the more highly evolved recognition receptors (i.e., antibodies and T cell receptors) are exquisitely specific for individual pathogenic species, more primitive recognition receptors capable of binding to multiple pathogens have been shown to contribute to host defense in the early phases of infection (1, 2). Several of these molecules have been shown to recognize carbohydrates contained in the cell walls of pathogens such as yeast and bacteria (2, 3). Because these carbohydrates are not expressed in vertebrate cells, their specific recognition provides a crude discrimination between self and nonself. Thus, acute-phase reactants capable of binding to α-mannan expressed in the cell walls of pathogens such as candida, aspergilli, mycobacteria, and pneumocystis have been postulated to contribute to early host defense against these pathogens (3, 4). Similarly, phagocytic cells such as monocytes and polymorphonuclear leukocytes express β-glucan receptors that allow the recognition and phagocytosis of pathogenic yeast (5).

Natural killer cells are also involved in the early phases of host defense. NK cells recognize and kill certain tumor cells and virally infected cells in a non-MHC-restricted manner, in the absence of prior sensitization (6). Although the surface molecules used in target cell recognition have not been fully characterized, recent results suggest that NK cells may express several, non-clonally distributed surface receptors. Just as soluble, pathogen-specific lectins have been shown to contribute to the humoral immune re-

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sponse, NK cell receptors possessing lectin domains are likely to contribute to the cellular immune response. Indeed, a family of NK-restricted surface molecules possessing lectin domains (e.g., NKR-P1, Ly-49, NKG2) has recently been identified (7–13). The observation that mAbs reactive with these cell surface lectins trigger NK cell killing suggests that these molecules might function as pathogen receptors. NK cells also express the CD11b/CD18 integrin that has been reported to be involved in the phagocytosis of yeast by neutrophils (14–16). The observation that CD11b/CD18 binds to the β-glucan component of the yeast cell wall suggests that this molecule might also be involved in the recognition of pathogens by NK cells. Although CD11b/CD18 has not been shown to be directly involved in NK killing (17), interaction of barley β-glucan with CD11b/CD18 on NK cells has been shown to enhance NK killing, suggesting that the lectin-like properties of this integrin molecule might facilitate NK cell activation. Another β-glucan-binding lectin has been identified on the surface of human monocytes (18). Molecular characterization of this β-glucan-binding lectin suggests that it is distinct from CD11b/CD18 (5). Here we report that human NK cells also express a β-glucan-binding cell surface lectin other than CD11b/CD18. Our results suggest that this β-glucan-binding receptor may be involved in NK-cell-mediated natural cytotoxicity.

MATERIALS AND METHODS

Reagents

Zymosan, particulate and soluble β-glucan, pustulan, and epoxy-activated Sepharose 4B were obtained from Sigma Chemical Co. (St. Louis, MO). One-micrometer-diameter polystyrene beads were obtained from Polyscience, Inc. (Warrington, PA).

Cells

NK cells were isolated from the peripheral blood of healthy volunteer donors as follows. Peripheral blood mononuclear cells were isolated by centrifugation over Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). After depletion of monocytes by a 1-hr adherence to plastic dishes, cells were depleted of T cells, B cells, and residual monocytes by magnetic bead depletion (Dynal, Oslo, Norway) using monoclonal antibodies reactive with CD5 (24T6G12, IgG2), CD3 (RW24B6, IgG1), CD20 (B1, IgG2), and CD14 (My4, IgG2) as previously described (19). The resulting cells were phenotypically <5% $CD3^+$, 75–95% $CD56^+$, and 65–80% $CD16^+$ as determined by flow cytometric analysis using an Epics profile (Coulter, Hialeah, FL).

Cell lines (YT, Molt-4, K562, U937, Daudi) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum in a humidified incubator containing 5% CO_2.

Cytolytic Effector Assays

^{51}Cr-labeled target cells were combined with NK cells at the indicated effector:target ratios using 3×10^3 targets per V-bottom microtiter plate in triplicate. Spontaneous lysis was determined by incubation targets in medium alone, whereas maximum lysis was determined by incubating targets in medium containing 1% Nonidet-P40 (Sigma Chemical Co.). Microtiter plates were centrifuged at 600 rpm for 2 min and then incubated for the indicated times at 37°C. Plates were then harvested using a Scatron

harvester and assayed for released ^{51}Cr as previously described (20). When particulate zymosan, glucan, or polystyrene was added to cytolytic effector assays, particle counts were determined by microscopic inspection in a hemocytometer. After addition of the indicated particle:NK cell ratios, assays were carried out as described above.

NK Cell Proliferation Assays

Freshly isolated NK cells were cultured in triplicate samples in round-bottom 96-well microtiter plates (Intermed., Denmark) containing 5×10^4 cells/well in RPMI 1640 containing 10% fetal calf serum. After addition of the indicated ratio of zymosan, NK cells were incubated for the indicated times in the absence or presence of recombinant human IL-2 (100 IU/ml, Genzyme, Inc. Cambridge, MA) prior to pulsing with [^{3}H]thymidine (1 μCi/well) (New England Nuclear, Boston, MA) for 8 hr. Cells were then harvested using a PHD cell harvester apparatus, and [^{3}H]thymidine incorporation was measured using a Packard liquid scintillation counter. Reported results represent the means of triplicate determinations in which standard errors were <15%.

Radioiodination and Precipitation of β-Glucan-Binding Lectins

Freshly isolated NK cells and the indicated tumor cell lines (YT, K562, Molt-4, and U937) were iodinated using ^{125}I (1 mCi/1 $\times 10^7$ cells) by the lactoperoxidase method (21). After washing three times to remove free iodide, cells were solubilized in NP-40 lysis buffer (1% NP-40, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* PMSF) for 30 min on ice and centrifuged at 12,000 rpm in a microfuge for 30 min at 4°C. Supernatants were precleared twice using protein A–Sepharose beads and then further incubated with either zymosan particles (25 μl packed particles), Sepharose beads alone, or Sepharose beads coupled to either mannan or pustulan (25 μl packed beads each) for 2–4 hr at 4°C with constant agitation. After washing four times with NP-40 lysis buffer, samples were eluted into sample buffer (2% SDS, 10% glycerol, 0.1 *M* Tris–HCl, pH 6.8, 0.02% bromphenol blue) and separated on a 15% SDS–polyacrylamide gel. Dried gels were subjected to autoradiography using Kodak XAR film.

RESULTS

Inhibition of Natural Cytotoxicity by Zymosan and Glucan Particles

Zymosan and glucan particles derived from the cell walls of yeast have been shown to have profound immunomodulatory properties (22). Although these biological effects are primarily directed at mononuclear phagocytes, the demonstration that a pathogenic yeast, *Candida albicans,* could inhibit NK cell lysis of K562 cells suggested that some component of the yeast cell wall might also affect NK cell function (23). We therefore measured the ability of freshly isolated peripheral blood NK cells to lyse K562 targets in the presence of either zymosan or glucan particles. Figure 1A shows the dose-dependent inhibition of NK killing by zymosan particles at various E:T ratios. Whereas maximum inhibition by zymosan occurred at a zymosan:NK cell ratio of 500:1, significant inhibition of NK killing occurred at a zymosan:NK cell ratio of 50:1. In other experiments not shown, the optimal zymosan:NK cell ratio was found to be 150:1. Particulate yeast glucan, a cell wall extract composed primarily of 1,3-β-D-glucan (24), similarly inhibited NK killing in a dose-dependent manner (Fig. 1B). Neither zymosan nor particulate glucan affected the viability of NK cells at any of the concentrations

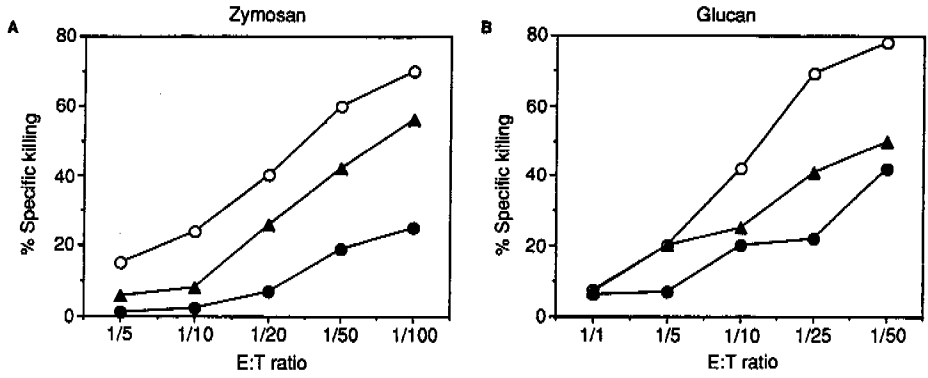

FIG. 1. Inhibition of natural cytotoxicity by zymosan and particulate β-glucan. (A) ^{51}Cr-labeled K562 cells were combined with freshly isolated NK cells at the indicated E:T ratios in the absence or presence of zymosan particles. After a 4-hr incubation in U-bottom 96-well microtiter plates, supernatants were analyzed for the release of ^{51}Cr as described under Materials and Methods. The zymosan:NK cell ratio is 0 (open circles), 50 (closed triangles), and 500 (closed circles). (B) An identical analysis was performed using particulate β-glucan in place of zymosan. Here the glucan:NK cell ratios are 0 (open circles), 50 (closed triangles), and 150 (closed circles).

tested as judged by trypan blue exclusion. Control experiments showed that the decreased ^{51}Cr release in the presence of zymosan or particulate glucan did not result from binding of free ^{51}Cr to the yeast particles. These results suggest that a component of the yeast cell wall present in both zymosan and glucan is capable of interacting with either NK cells or K562 cells to inhibit cytolysis.

If the observed inhibition resulted from an interaction between zymosan and the target cell, we might expect different target cell types to differ in their susceptibility to zymosan. We therefore compared the inhibitory effect of zymosan on NK killing directed against a panel of tumor cell lines. As shown in Fig. 2, zymosan similarly inhibited NK cell killing of HL-60, K562, U937, and Molt-4. Interestingly, antibody-dependent cytotoxicity (ADCC) mediated by these same NK cells was relatively unaffected by zymosan (Fig. 3). This result is consistent with previous observations suggesting that NK killing and ADCC are triggered by distinct cell surface receptors (20, 25). The relative inability of zymosan to inhibit ADCC suggests that the inhibition of natural cytotoxicity is not a consequence of steric hindrance of NK cell:target cell interactions by zymosan particles. Further evidence that the inhibitory effect of zymosan was not due to a particulate barrier between NK cells and target cells was obtained by comparing the ability of zymosan and zymosan-sized (1 μm diameter) polystyrene beads to inhibit NK cell killing of K562 cells. At equivalent particle:NK cell ratios, zymosan inhibited NK cell killing, whereas zymosan-sized polystyrene particles did not (data not shown). These results suggest that the zymosan-mediated inhibition results from a specific interaction with NK cells.

Effect of Zymosan on NK Cell Proliferation

The ability of zymosan particles to similarly inhibit NK cell killing of each of the target cells tested suggested that the observed effect was mediated through a specific interaction with the NK cell. We therefore measured the effect of zymosan particles

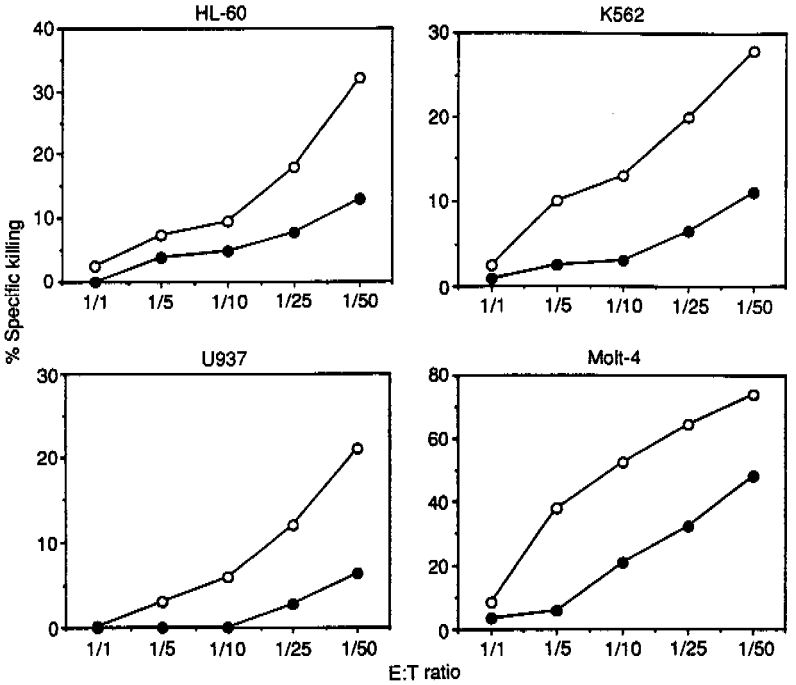

FIG. 2. Target cell specificity of the zymosan-induced inhibition of NK-cell-mediated cytotoxicity. Freshly isolated NK cells were combined with the ^{51}Cr-labeled target cells at the indicated E:T ratios in the absence (open circles) or presence (closed circles) of zymosan (zymosan:NK cell ratio = 150:1). After a 4-hr incubation at 37°C, supernatants were analyzed for the release of ^{51}Cr as described under Materials and Methods.

on NK cell proliferation in the absence or presence of IL-2. As shown in Table 1, zymosan particles alone induced a modest degree of NK cell proliferation (average SI = 4.4 ± 1.2, n = 4). In the presence of IL-2, however, NK cell proliferation was

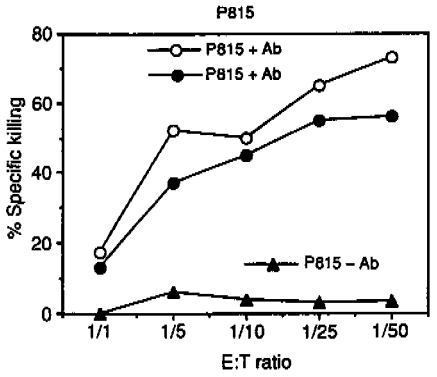

FIG. 3. Effect of zymosan on antibody-dependent cell-mediated cytotoxicity. ^{51}Cr-labeled P815 cells that had been preincubated with rabbit anti-P815 sera were cultured with freshly isolated NK cells at the indicated E:T ratios in the absence (open circles) or presence (closed circles) of zymosan (zymosan:NK cell ratio = 150:1). After a 4-hr incubation at 37°C, supernatants were analyzed for the release of ^{51}Cr. Closed triangles show ^{51}Cr release from P815 cells that had not been preincubated with rabbit antisera.

TABLE 1

Effect of Zymosan on NK Cell Proliferation[a]

Experiment 1

Stimulus	[^{3}H]Thymidine incorporation, cpm		
	Day 2	Day 4	Day 6
Media	560	1,409	2,899
Zymosan (1:5)	2,669	6,479	7,157
IL-2 (100 units/ml)	6,030	19,670	20,371
Zymosan + IL-2	4,911	7,596	5,382

Experiment 2

Stimulus	[^{3}H]Thymidine incorporation, cpm	
	Day 5	Day 7
Media	230	211
Zymosan (1:5)	667	702
IL-2 (100 units/ml)	7,898	5,789
Zymosan + IL-2	2,594	713

[a] Peripheral blood NK cells (40,000 cells/well) were incubated with the indicated stimuli for the indicated times before pulsing with [^{3}H]thymidine as described under Materials and Methods.

markedly inhibited in the presence of zymosan particles (Table 1). Thus, by acting as a weak agonist, zymosan somehow interferes with the stronger mitogenic signal triggered by IL-2.

Effect of Yeast Cell Wall Sugars on NK Killing

The major carbohydrate constituents of yeast cell wall zymosan are polymeric glucans (1,3-β-D-glucan and 1,6-β-D-glucan) and α-mannan (26). Particulate glucan is a more highly purified yeast cell wall derivative whose major constituents are 1,3-β-D-glucan and 1,6-β-D-glucan (24). The ability of both zymosan and glucan particles to inhibit NK killing suggests that one of the β-D-glucans is responsible for the observed effects. Since soluble β-glucan monomer has been reported to enhance NK killing (an effect attributed to the β-glucan-binding integrin CD11b/CD18), we measured the effect of β-glucan monomer on NK killing in the presence or absence of particulate zymosan. Although soluble β-glucan was able to slightly enhance NK killing, it had no effect on the zymosan-induced inhibition (data not shown). We similarly determined that linear 1,6-β-D-glucan (pustulan) did not affect the zymosan-mediated inhibition of NK killing at concentrations up to 500 μg/ml. These results suggest either that β-D-glucan must be presented in particulate form to affect NK killing or that another component present in zymosan particles but absent in pustulan is required for the observed inhibition.

The CD11b/CD18 integrin has been shown to interact with zymosan in neutrophils (15, 16). Since NK cells also express this complex, it was possible that the biological effects of zymosan were mediated through this molecule. We therefore measured the

effect of zymosan on NK killing in the presence or absence of an anti-CD11b mAb that has been shown to disrupt the zymosan:CD11b/CD18 interaction in neutrophils. As previously reported by others, anti-CD11b did not affect NK killing (17, and our unpublished data). The inhibitory effect of zymosan was also unaffected by this antibody. This result suggests that CD11b/CD18 may not be the NK cell surface molecule that binds zymosan.

Identification of β-Glucan-Binding Proteins on the Surface of NK Cells

The recent biochemical characterization of a β-glucan receptor on the surface of human monocytes (5) suggested the possibility that NK cells might also express a β-glucan receptor other than CD11b/CD18. We therefore used zymosan particles as affinity matrices for the identification of β-glucan-binding proteins expressed on the surface of NK cells. Because NK cells purified from peripheral blood mononuclear cells might be contaminated with monocytes, we performed our initial analysis using YT cells, an NK-like cell line capable of zymosan-inhibitable non-MHC-restricted killing (data not shown). Figure 4A shows that zymosan particles specifically precipitate proteins of approximately 12 and 16 kDa from detergent lysates derived from surface iodinated YT cells. Neither Sepharose beads alone nor Sepharose beads coupled to an irrelevant mAb precipitated these proteins. YT cells activated with either Con A or PHA/PMA appeared to express slightly more of these zymosan-binding proteins, suggesting that they may be induced during cellular activation. The 30-kDa zymosan-binding protein in Con A-activated YT cells is likely to be Con A itself which apparently binds to zymosan. Low-molecular-weight zymosan-binding proteins were similarly identified in purified peripheral blood NK cells as shown in the left panel of Fig. 4B. Whereas both YT cells and NK cells expressed these zymosan-binding proteins, NK targets such as U937 and K562 cells did not. Longer exposures of these same autoradiograms did, however, reveal the high-molecular-weight zymosan-binding proteins in U937 cells as previously described by Czop and Kay (5) (data not shown). Figure 4B also shows that pustulan-coupled Sepharose beads precipitated 12- and 16-kDa proteins from both YT cells and NK cells. Pustulan–Sepharose also precipitated several higher-molecular-weight proteins from NK cell lysates. None of these pustulan-binding proteins were precipitated from lysates prepared from U937 or K562 target cells. The specificity of these results is confirmed by the inability of either Sepharose beads alone or mannan-coupled Sepharose to precipitate similar proteins (Fig. 4B, left panels).

DISCUSSION

The ability of plant lectins such as concanavalin A and phytohemaglutinin to interact with lymphocyte activation receptors has long been appreciated. More recently, a number of lymphocyte surface molecules have been shown to possess lectin domains that afford them the potential to interact with surrounding carbohydrates (2, 27). In this capacity, cell surface lectins are able to mediate cell:cell and cell:matrix interactions. Lymphocyte receptors possessing lectin domains include the low-affinity Fc receptor for IgE (CD23) (28) and L-selectin (LAM-1) (29). Although the precise carbohydrate specificity of these molecules has not yet been determined, the importance of lectin: carbohydrate interactions in cellular trafficking through the reticuloendothelial system has been well documented (27, 30).

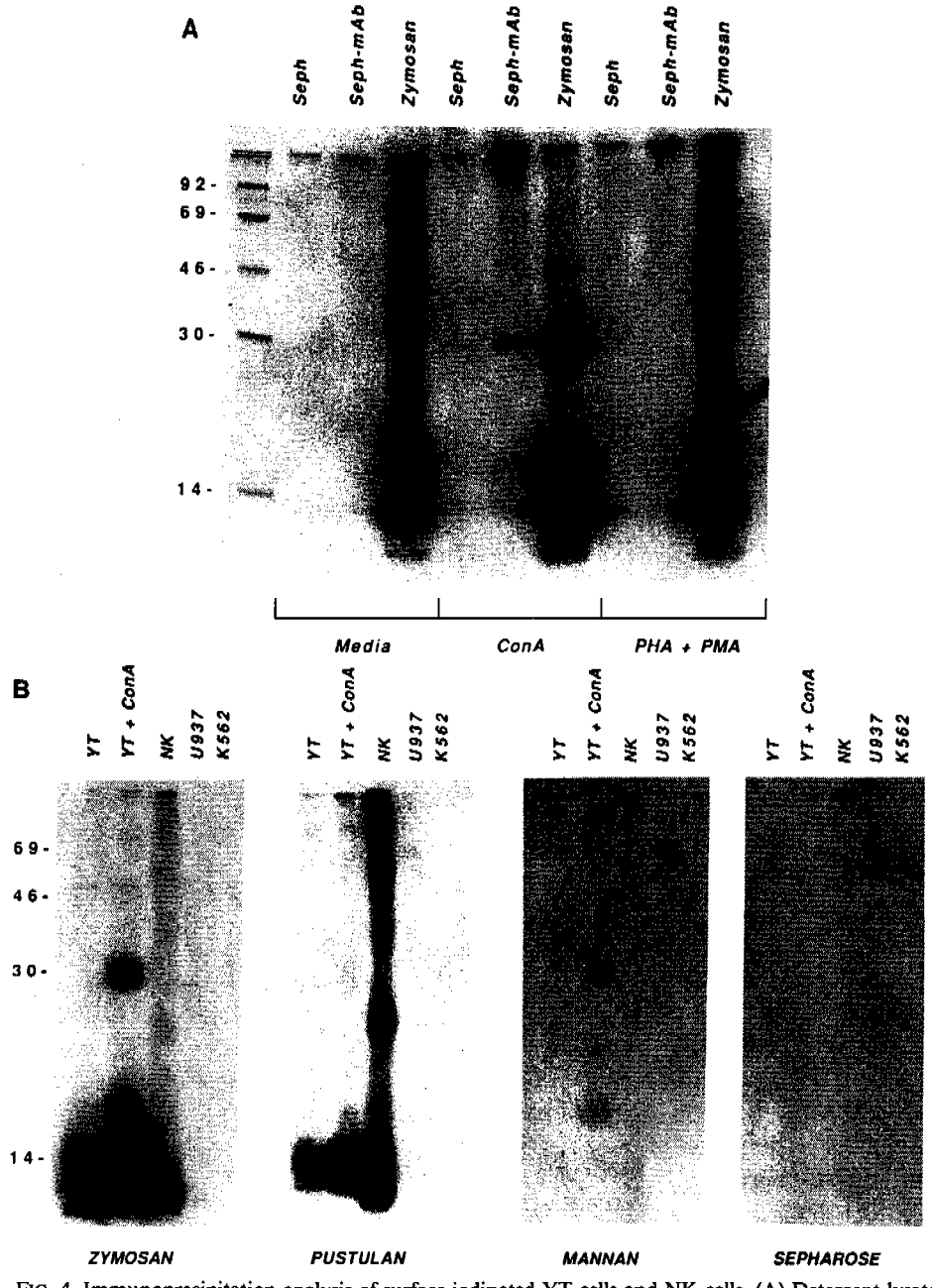

FIG. 4. Immunoprecipitation analysis of surface iodinated YT cells and NK cells. (A) Detergent lysates prepared from surface iodinated YT cells (resting or activated with either 25 μg/ml concanavalin A or 0.025 μg/ml phytohemagglutinin and 1 ng/ml PMA were immunoprecipitated with Sepharose beads alone (Seph), Sepharose beads coupled to a murine monoclonal IgG1 antibody (Seph-mAb), or zymosan particles (10 μl packed particles each) prior to electrophoretic separation on a 15% SDS–polyacrylamide gel under nonreducing conditions. The dried gel was subjected to autoradiography for 5 days. (B) Immunoprecipitates prepared from surface iodinated YT cells, Con A-activated YT cells, peripheral blood NK cells, U937 cells, and K562 cells were separated on a 12% SDS–polyacrylamide gel and analyzed by autoradiography as described above. Immunoprecipitates were prepared using zymosan particles (zymosan), pustulan-coupled Sepharose (pustulan), mannan-coupled Sepharose (mannan), or Sepharose beads alone (Sepharose) as indicated.

Recent results have shown that the NKR-P1 family of NK cell surface molecules possessing lectin domains have the potential to directly trigger cytolytic effector function (7, 10, 11). These observations suggest that carbohydrate molecules expressed on the surface of target cells might be involved in NK cell recognition. NK cells are thought to be a primitive, perhaps vestigial, component of the cellular immune system. Although they do not express the variable antigen receptor found on T cells, they are able to recognize certain transformed and virally infected cells. NK cells have also been reported to possess fungicidal (31, 32) and bacteriostatic (33, 34) properties, suggesting that they might also recognize certain unicellular pathogens. Since each of these targets express carbohydrates that differ from those found on normal vertebrate cells, lectin receptors expressed on the surface of NK cells could play a role in their specific recognition.

Our results show that particulate glucan and zymosan inhibit NK cell-mediated cytotoxicity. Since these yeast cell wall derivatives are composed primarily of β-D-glucan, this result suggests the possibility that a β-glucan-binding lectin might be involved in this process. Previous results have shown that barley β-glucan enhances NK killing (35). Because this effect could be blocked by anti-CD11b mAb, the NK cell integrin CD11b/CD18 was postulated to be responsible for this activity. Indeed, CD11b/CD18 has also been implicated in the phagocytosis of zymosan by neutrophils, suggesting the possibility that CD11b/CD18 is a β-glucan-binding lectin (14–16). However, differences in the ability of zymosan and particulate glucan to activate neutrophils suggest the possibility that another component of zymosan might be involved in this process (36). Furthermore, mAbs reactive with CD11b/CD18 do not affect NK cell killing (17). It is therefore possible that a β-glucan-binding NK cell surface molecule other than CD11b/CD18 might be responsible for some of the biologic effects of soluble and particulate β-glucans. It is clear that an improved understanding of NK cell surface lectins will increase our understanding of the recognition receptors used by this cytolytic effector cell. Consistent with this possibility, we have identified two prominent β-glucan-binding proteins of 12 and 16 kDa on the surface of NK cells and YT cells. Although our results do not prove that these molecules are responsible for the inhibitory effects of zymosan and particulate glucan, they warrant further study as candidate NK cell recognition receptors.

ACKNOWLEDGMENTS

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