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ScienceDirect
Nutrition Research 27 (2007) 327–335
Nutrition Research
www.elsevier.com/locate/nutres

An antiinflammatory immunogen from yeast culture induces activation and alters chemokine receptor expression on human natural killer cells and B lymphocytes in vitro

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Received 27 November 2006; revised 11 April 2007; accepted 13 April 2007

Abstract

The aim of this study was to evaluate the immunomodulating effects of a consumable yeast-based immunogen, EpiCor, on human leukocytes in vitro. The selection of antiinflammatory and lymphocyte activation assays was based on initial evidence for immunomodulating effects of EpiCor from an unusually low incidence of influenza among employees in a factory manufacturing EpiCor, along with a high oxygen radical absorbance capacity value. In the present study, EpiCor significantly reduced the production of reactive oxygen species by neutrophils ($P < .005$). EpiCor treatment of peripheral blood mononuclear cells (PBMCs) caused induction of the activation markers CD80 and CD86 on B lymphocytes, and CD69 and CD25 on $CD3^{-}CD56^{+}$ natural killer cells. This induction was also seen on enriched populations of natural killer and B lymphocytes, suggesting a direct effect not dependent on bystander cells. Coculturing of PBMC with EpiCor and phytohemagglutinin resulted in inhibition of phytohemagglutinin-induced T-cell proliferation and reduction of interferon gamma production. Fucoidan, a ligand for the homing molecule L-selectin (CD62L), is known to induce rapid up-regulation of several chemokine receptors on lymphocytes. EpiCor caused strong inhibition of Fucoidan-mediated expression of the chemokine receptors CXCR4 and CCR9 on PBMC. This suggested rapid altering of signal transduction pathways, or a direct competition for cell surface receptors, with an end result being an altered sensitivity to chemotactic signals from tissue. We conclude that EpiCor possesses significant antiinflammatory activity and induces direct activation and increased chemotactic awareness of lymphocyte subsets in vitro. This suggests further study of effects of EpiCor consumption on antiviral defense mechanisms and antibody production.

Keywords: *Saccharomyces cerevisiae*; Human; Immune; NK cell; B cell; Chemokine receptor; Interferon; ROS; Antiinflammatory

1. Introduction

EpiCor is an immunogen product based on anaerobic fermentation of baker's yeast (*Saccharomyces cerevisiae*) in a proprietary medium. After fermentation, the whole liquid is dried, resulting in a product that is high in yeast metabolites, including vitamins, polyphenols, sterols, and phospholipids. An unusually low incidence of influenza infectivity among certain employees over several decades working in a fermentation facility in Cedar Rapids, Iowa, led to a theory that these employees' exposure to a yeast-based product produced at the facility and absorbed daily by inhalation and/or ingestion might have an effect on their

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doi:10.1016/j.nutres.2007.04.008

immune defense. Bioactive components include the nutrient/vitamin profile, cell wall components, and stress-induced metabolites. Based on the known effects of yeast cell walls on immune cell reactions in vitro, an overall proinflammatory effect could be expected. However, the product has an oxygen radical absorbance capacity (ORAC) value within the range of 450 to 650 μmol Trolox equivalents per gram [1] and a high reactive oxygen species (ROS) scavenging activity [2]. The batch of EpiCor used in this study had an ORAC value of 614 μmol of TE (trolox equivalents) per gram. The overall combined effects of the complex profile of pro- and antiinflammatory compounds present in EpiCor have not previously been studied on human immune cells in vitro. The decrease in incidence of viral disease among the facility workers and the extremely high antioxidant value of this food prompted this study to assess for antiinflammatory potential and direct effects of EpiCor on different lymphocyte subsets in vitro.

Among the known immunomodulatory compounds from *S. cerevisiae*, various cell wall compounds have been studied. The primary compound of the inner layer of cell wall is β-1-3-glucan, interspersed with some β-1-6-glucan, whereas the outer part of the wall is mostly composed of mannans. Glucans are present in many plants and all fungi. β-Glucans are known immunomodulators, and in vitro and animal works indicate that β-glucan supports a shift toward Th1 responsiveness [3]. Mice that received oral β-glucan for 1 week before infection with anthrax showed dramatically increased survival [4]. Orally administered β-glucans have shown to protect against cancer in different experimental models [5,6]. Of interest to our data is the previous report on mouse spleen cells that particulate β-glucan from *S. cerevisiae*, in the presence of a strong mitogenic stimulus, results in reduced lymphocyte proliferation and interferon gamma (IFN-γ) production [7]. Mannans are known as immunomodulators [8] and as potent activators of natural killer (NK) cells [9]. Different mannans interact with distinct toll-like receptors (TLRs) because mannan from both *S. cerevisiae* and *Candida albicans* is recognized by TLR-4, whereas phospholipomannan is recognized by TLR-2 [10]. This allows for multifacetted immunomodulating events in immune cells treated with a complex product such as EpiCor. However, these cell wall components are likely only minor contributing factors to EpiCor's overall effects because of the high amounts of metabolites present in the product.

In this study, the effects of EpiCor were evaluated in a broad panel of in vitro cell-based assays, using different subsets of human leukocytes. Our hypothesis was that we would observe NK-cell activation and antiinflammatory effects in vitro. The antioxidant and antiinflammatory properties were tested on human neutrophils and erythrocytes in an assay aimed at demonstrating inhibition of formation of ROS. The immunomodulating properties were evaluated by modulation of phytohemagglutinin (PHA)-induced T-cell proliferation, demonstration of direct induction of calcium flux in lymphocyte subsets, and induction of activation markers by EpiCor. The data indicate potent antiinflammatory and antioxidant properties associated with this food product, in combination with activation of cells from the innate and adaptive immune response, particularly NK-cell activation, which may help explain the reduction in viral disease, such as influenza, seen in vivo.

2. Methods and materials

2.1. Study design

This study was conducted from February 2004 to June 2006 at NIS Labs, Klamath Falls, Ore. The study protocol was designed in part based on data from employee health records from a plant that produces EpiCor, showing unusually low incidences of flu and other viral diseases among employees with the highest daily exposure to EpiCor. A follow-up study compared exposed and unexposed employees, and showed increased ROS scavenging activity and NK-cell activity and increased salivary IgA among exposed employees [2]. Our in vitro study was therefore designed to examine whether the specific mechanisms of action of EpiCor included direct NK- and B-cell activation and, at the same time, showed effects on ROS inhibition.

2.2. Preparation of EpiCor for in vitro work

The EpiCor product was supplied from Embria Health Sciences, Cedar Rapids, Iowa, as dark brown flakes. A solution was prepared in phosphate-buffered saline (PBS) in preparation for in vitro cell-based assays by adding 0.5 g of powder to 5 mL of PBS. This mixture was vortexed for 30 seconds and allowed to sit at room temperature for 1 hour. This allowed most of the powder to dissolve, giving the PBS a dark brown color. The remaining solids were removed by centrifugation at 2400 rpm for 10 minutes and subsequent sterile filtration of the supernatant using a 0.22-μm syringe filter. A 10-fold dilution of the stock solution was prepared in RPMI and incubated at 37°C for 1 hour. This resulted in the formation of a white fluffy mineral precipitate, which was removed by filtration before adding the extract to cell cultures. The precipitate formed in RPMI but not in PBS. Initial tests comparing bioactivity of the extract before and after removal of the precipitate showed no differences in terms of NK-cell activation. The precipitate was removed in all subsequent experiments to avoid interference with equipment readings, including absorbance and flow cytometry.

2.3. Reagents and monoclonal antibodies

The following human lymphocyte-specific monoclonal markers directly conjugated with fluorochromes were purchased from Becton-Dickinson, San Diego, Calif: CD3-PerCP, CD14-PE, CD25-FITC, CD45-FITC, CD56-FITC, CD56-PE, CD69-FITC, CD80-PE, and CD86-PE. Antibodies for CXCR4 and CCR9 were obtained from R&D

Systems, Inc, Minneapolis, Minn. The anti-CD19 B-cell marker B4-FITC was purchased from Coulter, Hialeah, Fla. Buffers including RPMI-1640, Histopaque 1119 and 1077, and PBS without calcium or magnesium were from Sigma-Aldrich, St Louis, Mo.

2.4. Purification of peripheral blood mononuclear cells and polymorphonuclear cells

Peripheral venous blood samples were obtained, after informed consent, from healthy human volunteers between 20 and 60 years old, as approved by the Merle West Medical Center institutional review board. Samples were drawn into sodium heparin and processed within 30 minutes by gradient centrifugation [11,12]. In brief, whole blood was layered onto a double gradient of Histopaque 1119 and 1077, and centrifuged for 25 minutes at 400*g*. The upper peripheral blood mononuclear cell (PBMC)–rich interface and the lower polymorphonuclear (PMN) cell–rich interfaces were harvested using sterile pipettes, transferred to sterile vials, and washed twice in PBS at 2400 rpm for 10 minutes. After the second wash, the PMN cell pellet was treated with 4 mL of nanopure water and gently mixed by swirling for 30 seconds to lyse any remnant red blood cells. Immediately, 4 mL of 1.8% saline was added to restore physiologic isotonic strength. Four milliliters of PBS was added, and the PMN cells were washed for 10 minutes at 2400 rpm.

2.5. Assessment of ROS formation

The assessment of antiinflammatory capacity was performed in a cell-based system [13] using freshly purified PMN cells to examine whether treatment with a test product led to reduced oxidative damage during an oxidation challenge. The cells were pretreated with EpiCor and loaded with the precursor dye DCF-DA (cat. no. C6827; Molecular Probes, Eugene, Ore), which becomes brightly green fluorescent after exposure to oxygen free radicals. Subsequently, oxidative damage was triggered by addition of H_2O_2. Parallel samples of freshly purified human PMN cells were preincubated with EpiCor over a range of dilutions from 10 to 0.01 μL/mL of the aqueous extract, prepared as described hereinabove, at 37°C and 5% CO_2 for 90 minutes. A stock solution of DCF-DA was prepared by adding 180 mL of DMSO and 20 μL of a 20% solution of Pluronic F-127 in DMSO to a 50-μg aliquot of DCF-DA, and vortexing 3 times for 15 seconds. A working solution of DCF-DA was then prepared by adding a 10-μL stock to 10 mL of PBS. The cells were washed twice in PBS and then resuspended in the DCF-DA working solution, and incubated for 1 hour at 37°C. All samples, except for the untreated control samples, were then exposed to 167 mmol/L H_2O_2 for a period of 45 minutes to induce severe oxidative stress. Samples were washed twice in PBS to remove the peroxide, transferred to cold RPMI, and stored on ice in preparation for immediate analysis by flow cytometry, using a FacsCalibur flow cytometer (BD Biosciences, San Jose, Calif). Intracellular levels of DCF-DA fluorescence intensity in untreated vs challenged cells, in the presence vs absence of EpiCor, were analyzed by flow cytometry. Positive control samples were not pretreated with EpiCor to allow maximum ROS formation. Negative control samples were treated with neither EpiCor nor H_2O_2 to establish baseline levels of fluorescence in the samples. Two complete sets of positive and negative controls were used in all experiments, where flow cytometric analysis was performed on the first set of controls at the beginning and the second set of controls at the end of each set. This was to test for spontaneous changes in oxidation in samples over the course of the flow cytometry, ensuring that a reduced fluorescence seen with EpiCor samples was not a result of changes in the samples. Data were collected in triplicate for controls and duplicate for each sample concentration. Data analysis was performed using the CellQuest Pro (BD Biosciences) and FlowJo (TreeStar, Ashland, Ore) software packages. The mean fluorescence intensity (MFI) of PMN cells was compared among untreated, H_2O_2-treated, and EpiCor-treated samples. A reduction in MFI in samples pretreated with EpiCor before challenge with H_2O_2 signified a reduction in ROS production, as a result of antioxidant and antiinflammatory effects.

2.6. Enrichment of NK and B lymphocytes using RosetteSep

To test whether the effect of EpiCor was the result of direct activation of specific cell subsets, we took blood samples and immediately treated it with RosetteSep reagents from StemCell Technologies Inc, Vancouver, BC, Canada, to enrich for certain cell types [14]. For B-cell purification, the B-cell enrichment cocktail was used (cat no. 15024), and for NK cells, the NK enrichment cocktail was used (cat no. 15025). The RosetteSep kits are cocktails of antibodies directed at all the unwanted cell subsets in the blood sample and assist with the cross-linking of these cells to red blood cells. Upon treatment with the antibodies, the blood was layered onto Histopaque 1077 and centrifuged as described above. The interface consisted primarily of either NK (90% purity) or B cells (95% purity). Cells were washed twice in PBS with 10% autologous serum and used for culturing with EpiCor to assess induction of activation markers.

2.7. Induction of cell surface markers and immunostaining

Freshly purified PBMCs were resuspended in culture medium and exposed to serial dilutions over a range of dilutions from 10 to 0.01 μL/mL of the aqueous extract of EpiCor, for 18, 24, and 48 hours for NK- and T-cell activation, and for 1, 3, and 7 days for B-cell activation. Cells were harvested into V-bottom 96-well plates (Nunc, Denmark) and washed in IF buffer (PBS containing 1% bovine serum albumin and 0.02% sodium azide). Cells were resuspended in 50 μL of IF (immunofluorescence) buffer, and monoclonal antibodies were added in previous-

ly established optimal amounts (CD45-FITC, CD14-PE, CD3-PerPC, CD56-PE, CD69FITC, and CD25-FITC: 8 μL/sample; CD19-FITC: 2 μL/sample; CD80 and CD86-PE: 10 μL/sample) and incubated in a dark at room temperature for 10 minutes. An additional 110 μL of buffer was added to each well, and the plates were centrifuged. The supernatants were removed, and the cells were resuspended in IF buffer and transferred to aliquots of 0.4 mL of 1% formalin in PBS. Samples were stored dark and acquired by flow cytometry within 24 hours using a FacsCalibur cytometer (Becton-Dickinson, San Jose, Calif). Analysis was performed using the software Flow Jo (Tree Star Inc, Ashland OR). For evaluation of NK-activation markers, electronic gating was performed on forward and side scatter properties to include all lymphocytes while excluding nonviable cells as well as monocytes. Subsequently, gating excluded $CD3^+$ cells and included $CD56^+$ cells for final analysis of the 2 activation markers CD69 and CD25 on $CD3^-CD56^+$ NK cells. For analysis of B-cell activation markers, electronic gating on forward and side scatter excluded nonviable cells and small lymphocytes, but included all larger cells and lymphoblasts. Gating on $CD19^+$ B cells within the population of large lymphoblasts was performed, and then analysis of CD80 and CD86 expression on lymphoblasts, in proportion to all lymphocytes, was performed.

2.8. Cell proliferation assay

Freshly purified PBMCs were stained with a kit containing a lipophilic membrane dye PKH26 (Sigma-Aldrich), which is incorporated into the lipid bilayer of the cell membrane in a highly stable manner and is distributed between daughter cells at each mitotic division in culture [15]. The staining was performed according to the manufacturer's guidelines. In brief, PBMCs were resuspended in diluent C supplied with the kit and rapidly added to the PKH26 suspension. Cells were mixed by constant gentle pipetting for 30 seconds and then gentle agitation of the vial for an additional 4.5 minutes. The incorporation of dye was stopped by addition of an equal volume of serum, followed by dilution and repeated washing in RPMI containing 10% fetal calf serum. The labeled cells were resuspended at a concentration of 10^6/mL in culture medium and aliquoted into sterile round-bottom 96-well cell culture plates (Nunc). Triplicate sets of samples were prepared for each treatment, including various dilutions of EpiCor, spanning a range of dilutions from 10 to 0.01 μL/mL of the aqueous extract, with and without known stimuli in parallel sets of samples. As known stimuli, we used the mitogen PHA (2 μg/mL) in parallel to human recombinant IL-2 (50 IU/mL). Untreated samples served as negative controls. The plates were incubated for 5 days at 37° C at 5% CO_2. Cells were transferred from the culture plate to a V-bottom 96-well plate and washed twice with PBS. The pellets were resuspended in 50-μL PBS and transferred to 0.4 mL of 1% formalin in PBS. Data acquisition was performed on a FacsCalibur flow cytometer (Becton-Dickinson, San Jose, Calif). Analysis was performed using the software FlowJo (TreeStar). Electronic gating was performed on forward and side scatter properties to exclude nonviable cells, and analysis determined the proportion of cells remaining in the parent population as reflected by their fluorescence intensity.

2.9. Interferon gamma production

Interferon gamma is a proinflammatory cytokine often associated with NK-cell activation. The effect of EpiCor on IFN-γ production in conjunction with 2 known stimuli (PHA and IL-2) was examined. Freshly purified PBMCs were resuspended at 10^6/mL in culture medium and aliquoted into sterile round-bottom 96-well plates (Nunc). Serial dilutions of EpiCor, with and without addition of the mitogen PHA vs interleukin 2, were added to the wells, with each treatment being performed in triplicate. Doses of EpiCor, PHA, and IL-2 were added as described above. The plates were cultured for 5 days at 37°C at 5% CO_2. The supernatants were collected and assessed for content of IFN-γ using the DuoSet ELISA kit from R&D Systems. The absorbance was read at 450 nm, blanking on 570 nm, using a PowerWave X microplate reader (BioTek Instruments, Winooski, Vt). Data were exported to Excel for statistical analysis.

2.10. Induction of chemokine receptor expression by Fucoidan

Fucoidan is an L-selectin ligand that triggers a rapid expression of the chemokine receptor CXCR4 on human lymphocytes [16]. The relative expression of different chemokine receptors dictates the potential for a chemotactic response involved in lymphocyte recruitment to tissue. To evaluate whether EpiCor would alter the chemokine

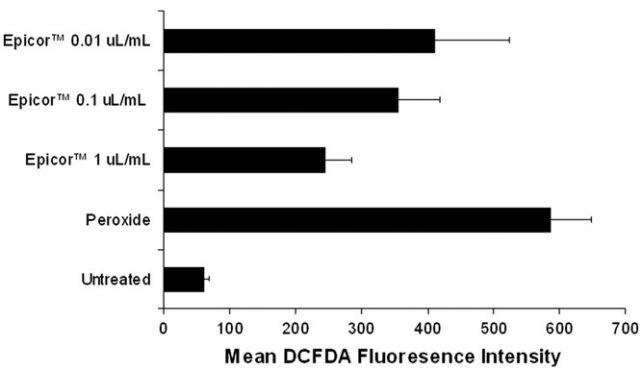

Fig. 1. The antiinflammatory properties of EpiCor were quantified by measuring the inhibition of formation of intracellular ROS in human PMN cells. The PMN cells were pretreated with EpiCor and loaded with the ROS reporter dye DCF-DA before triggering formation of ROS by H_2O_2. Reactive oxygen species formation within the PMN cells was measured as MFI of the dye, which becomes green fluorescent upon exposure to ROS. The data shown are mean ± SD of triplicate tests and are representative of 4 similar experiments. A dose-dependent inhibition of ROS formation by EpiCor was seen. This ROS inhibition was highly significant at the highest dose of EpiCor at 1 μL/mL ($P < .005$).

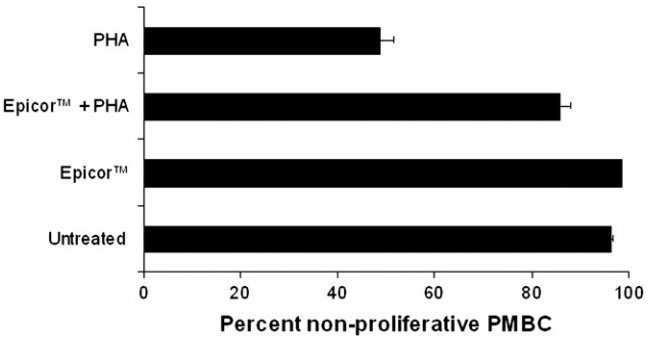

Fig. 2. EpiCor modulated the proliferative response of human PBMC to the known T-cell mitogen PHA. The costimulation of PBMC with PHA and EpiCor resulted in a significant increase of nonproliferating cells compared with PHA alone ($P < .00001$), indicating that compounds within EpiCor altered the cell signaling by PHA. EpiCor showed no direct mitogenic effect on human PBMC. Data are shown on triplicate samples from 1 experiment (mean ± SD, n = 3), representative of 3 experiments with cells from different individual donors.

receptor profile, we resuspended freshly purified PBMCs in RPMI 1640 and aliquoted it into microwell plates at a concentration of 2×10^5 cells/well, and then exposed it to Fucoidan (0.1 mg/mL). In parallel, PBMCs were exposed to either EpiCor alone (10 μL/mL) or a premixed cocktail of Fucoidan with EpiCor. The samples were incubated for 5 to 60 minutes, washed with IF buffer, and stained using PE-conjugated monoclonal antibodies specific for the CXCR4 and CCR9 chemokine receptors (R&D Systems) at 8 μL/sample. Untreated cells were used for assessment of baseline expression of CXCR4 and CCR9.

2.11. Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2003 (Microsoft Corp, Redmond, Wash). The averages of identical control vs test samples were calculated, and the statistical significance was tested using the independent sample *t* test, also known as Student *t* test [17,18]. A probability (*P*) value of less than .05 indicated that the averages between 2 groups of data were significantly different.

3. Results

3.1. Reduction of ROS in PMN cells by EpiCor

Human PMN cells were used for testing of the combined antioxidant and antiinflammatory effect of EpiCor. The PMN cells were pretreated for 90 minutes with EpiCor before loading with the oxidation-sensitive dye DCF-DA and the induction of reactive oxygen burst, which generates large amounts of intracellular free oxygen radicals. Untreated cells were assayed to establish the baseline level of oxidation of the indicator dye DCF-DA in the absence of an oxidative challenge. EpiCor pretreatment resulted in significant reduction of ROS formation when an oxidative burst was triggered in PMN cells challenged with hydrogen peroxide (Fig. 1). The effect was dose dependent, and at the highest dose of EpiCor, the effect was highly significant ($P < .005$). This assay does not distinguish between compounds with a direct antioxidant effect vs compounds mediating an antiinflammatory effect, which, by binding to cell surface receptors and altering signaling, leads to the reactive oxygen burst. However, the assay showed that, overall, EpiCor had a highly significant antiinflammatory effect in this cell-based system.

3.2. Modulation of PHA-induced lymphocyte proliferation by EpiCor

The proliferative response to the T-cell mitogen PHA was used to compare untreated PBMC to PBMC exposed to EpiCor for 5 minutes before exposure to PHA. No proliferative response was induced in response to EpiCor, indicating that EpiCor was not in itself mitogenic for human

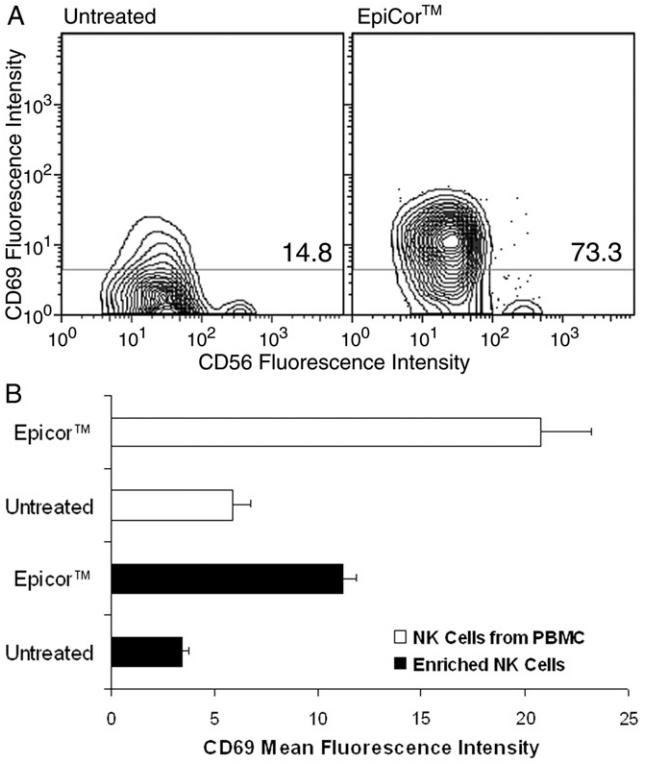

Fig. 3. Flow cytometry analysis showed that EpiCor caused expression of the NK activation marker CD69. (A) Induction of the CD69 activation marker on NK cells from PBMC cultures after 18 hours of incubation in the presence vs absence of EpiCor (10 μL/mL) was evaluated by multicolor immunofluorescence, where electronic gating allowed analysis of CD69 on $CD3^-CD56^+$ NK cells. The data shown are based on samples from 1 of 3 representative experiments. The data show that CD69 was induced by EpiCor on the $CD56^{medium}$ cells, but not on the $CD56^{bright}$ cells. (B) EpiCor-mediated induction of the CD69 activation marker on human NK cells was seen both on NK cells from PBMC cultures and on enriched NK cells. The induction of CD69 was statistically significant both on NK cells from PBMC cultures ($P < .03$) (white bars) and from enriched NK-cell cultures ($P < .02$) (black bars). Data are shown on triplicate samples from one experiment (mean ± SD, n = 3). The induction of CD69 was lower on purified NK cells than on mixed PBMC, indicating that other cells within the PBMC may further enhance the NK-activating effect of EpiCor.

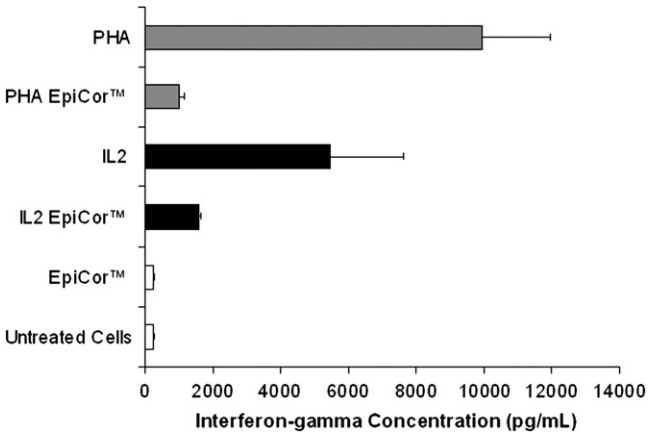

Fig. 4. Inhibition of IFN-γ production EpiCor. Human PBMCs were incubated for 5 days in the absence vs presence of EpiCor (10 μL/mL) with and without PHA or IL-2. Culture supernatants were harvested and assayed for IFN-γ by enzyme-linked immunosorbent assay. The data shown are mean ± SD (n = 3). When comparing the level of IFN-γ production as a result of PHA alone vs PHA + EpiCor treatment, a statistically significant inhibition was seen ($P < .02$). When comparing the level of IFN-γ production as a result of IL-2 alone vs IL-2 + EpiCor treatment, an inhibition was seen but did not meet the criterion for significance ($P < .09$).

lymphocytes (Fig. 2). However, proliferation was significantly reduced in the presence of EpiCor ($P < .00001$) compared with PHA alone, indicating that EpiCor was engaging signaling components, so subsequent signaling by PHA was modulated on human T lymphocytes.

3.3. Induction of the CD69 activation marker on NK cells by EpiCor

Evaluation of activation of NK cells was examined using 18-hour culturing followed by immunostaining for NK- and T-cell lineage markers in combination with the activation markers CD69 and CD25 (the IL-2 receptor). EpiCor treatment resulted in the induction of CD69 on $CD3^{-}CD56^{+}$ NK cells ($P < .03$) (Fig. 3). The induction of CD69 on NK cells was strictly limited to those NK cells with a moderate expression of CD56 and was not observed on the small subset of $CD56^{bright}$ NK cells (Fig. 3A). The increase in CD69 expression was of similar proportions when examining the NK cells in PBMC cultures by electronic gating on $CD3^{-}CD56^{+}$ cells and when NK cells were physically purified by the RosetteSep method (Fig. 3B). These highly enriched NK cells also displayed significant up-regulation of the CD69 marker when exposed to EpiCor ($P < .02$), meaning that the NK-activating effect of EpiCor was at least in part caused by a direct activation of NK cells and not dependent on bystander cells.

3.4. Inhibition of PHA-induced and IL-2–induced IFN-γ production by EpiCor

Interferon gamma is a proinflammatory cytokine made by certain cell types including activated NK cells. Peripheral blood mononuclear cell culture supernatants from 5-day cultures were tested for IFN-γ production in the presence vs absence of EpiCor. Two known stimulators of IFN-γ production, PHA and IL-2, were used alone and in combination with EpiCor. EpiCor treatment of PBMC reduced the levels of PHA-induced ($P < .02$) as well as the IL-2–induced ($P < .09$) IFN-γ production (Fig. 4). EpiCor alone did not induce IFN-γ production.

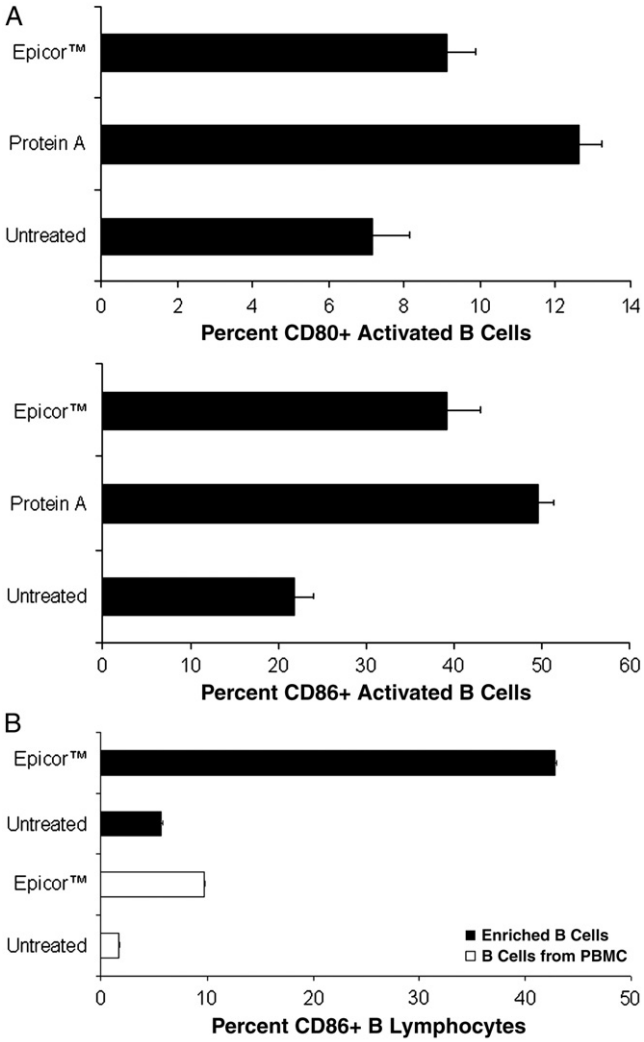

Fig. 5. EpiCor-mediated activation of B cells. (A) The expression of CD80 and CD86 on large B lymphoblasts was evaluated by immunostaining and flow cytometry. The induction of these 2 B-cell activation markers was greater in cultures treated with EpiCor (10 μL/mL) than in cultures where B cells were activated with *Staphylococcus aureus* protein A (protein A, 10 μg/ml). The differences in activation marker expression between untreated and EpiCor-treated cells were statistically significant for both CD80 ($P < .0001$) and CD86 ($P < .02$). Data are shown on triplicate samples from 1 experiment (mean ± SD, n = 3) and are representative of 3 experiments with cells from different individual donors with similar results. (B) The EpiCor-mediated B-cell activation was a result of direct effect of EpiCor on B cells. Data are shown on triplicate samples from one experiment (mean ± SD, n = 3). White bars indicate the CD86 expression on B cells electronically gated from PBMC cultures based on their expression of the B-cell marker CD19. Black bars show the CD86 expression on enriched B cells. The induction of CD86 on enriched B cells was highly significant ($P < .00001$).

These data showed that even though EpiCor activated NK cells, it did not lead to production of the proinflammatory cytokine IFN-γ, and it reduced IFN-γ production as a result from other stimuli.

3.5. Induction of activation markers on human B cells by EpiCor

Based on previous data showing that in vivo effects of EpiCor included increased salivary IgA production, it was speculated that a direct effect of EpiCor on B-cell activation was a key part of its mechanism of action. We showed that EpiCor induced a significant expression of the 2 B-cell activation markers, CD80 and CD86, in vitro, with a maximal expression of CD80 ($P < .0001$) as well as CD86 ($P < .02$) on day 3 of culture (Fig. 5A). As a positive control for B-cell activation, protein A was used to induce B-cell activation. The coculturing of PBMC with EpiCor and protein A resulted in an enhanced B-cell activation when compared with either substance alone (Fig. 5A). To evaluate whether the effect of EpiCor on human B cells was a result of a direct activation or collaborative events requiring the presence of several cell types, the testing was repeated with highly enriched B cells. The B cells were enriched from PBMC using a RosetteSep antibody cocktail and gradient centrifugation, and cultured with EpiCor. A highly significant expression of CD86 ($P < .00001$) was observed in these cultures (Fig. 5B), indicating that at least in part the effect of EpiCor on B cells was direct.

3.6. EpiCor modulation of L-selectin–mediated expression of chemokine receptors on NK cells

The expression of adhesion molecules and chemokine receptors on lymphocytes determines their ability to respond to recruitment signals from tissue and is thus an important factor in immune surveillance. Peripheral blood mononuclear cell treated for 24 hours with EpiCor showed an almost complete loss of staining with the monoclonal antihuman L-selectin antibody TQ1, which is specific toward the ligand-binding area of human L-selectin (Fig. 6A). This significantly reduced binding of TQ1 to PBMC ($P < .0001$ at 24 hours) could indicate that L-selectin was lost from the cell surface as a result of EpiCor treatment, possibly as a result of shedding of the extracellular portion of L-selectin from the cell surface via protein kinase C–mediated cell activation. Alternatively, the reduced binding of TQ1 to the L-selectin could be a result of a direct competition for binding between the TQ1 antibody and compounds in the EpiCor extract. Because the binding of ligands to L-selectin is known to modulate the profile of chemokine receptors, thus, altering chemotactic potential during leukocyte trafficking, further work on chemokine receptors was undertaken. Fucoidan, a known ligand for L-selectin, was used to provoke externalization of intracellular reservoirs of the chemokine receptors CXCR4 and CCR9. EpiCor interfered with the Fucoidan-mediated

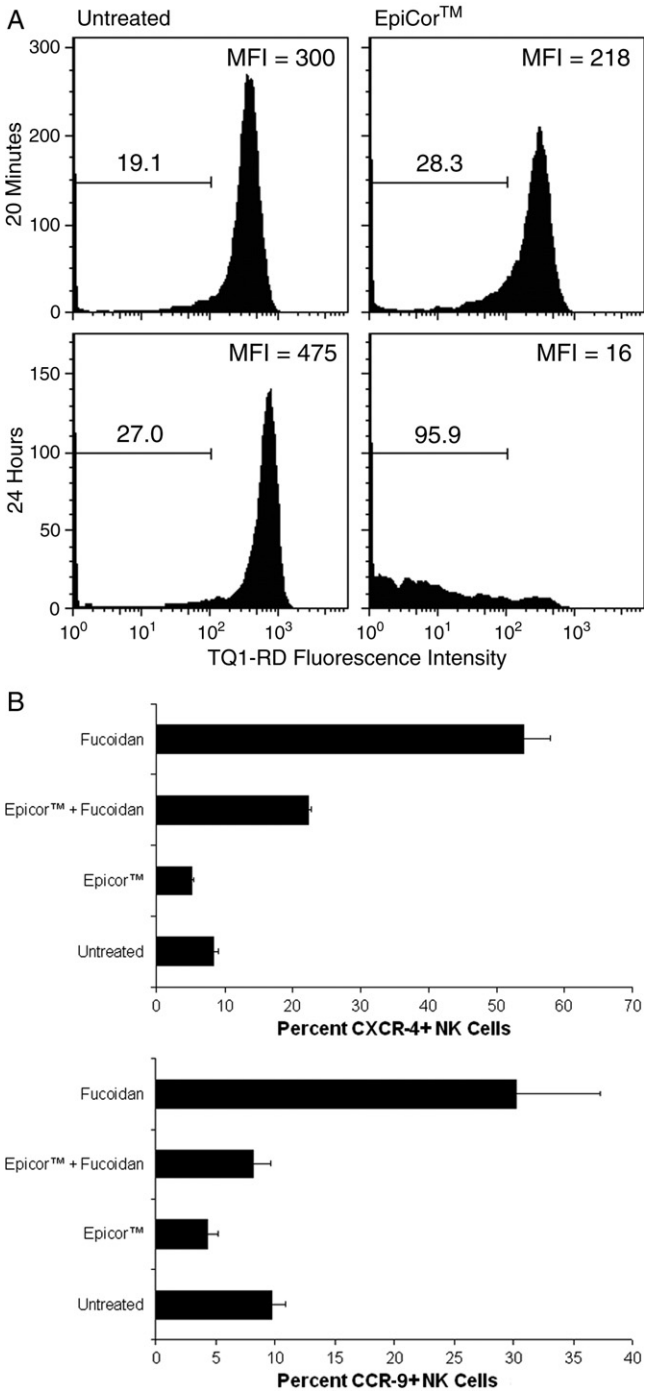

Fig. 6. EpiCor modulation of L-selectin–mediated expression of chemokine receptors on NK cells. (A) Binding of the monoclonal antibody TQ1, directed toward the ligand-binding area of the homing molecule L-selectin, was significantly reduced after exposure of lymphocytes to EpiCor (10 μL/mL) (24 hours, $P < .0001$). Data are representative of 3 similar experiments. (B) EpiCor served as an agonist for L-selectin–mediated chemokine expression on NK cells. When PBMCs were incubated with Fucoidan, a known L-selectin ligand, the cells rapidly (within minutes) and transiently externalized the chemokine receptors CXCR4 and CCR9 many-fold above background expression level. Brief 10-minute preincubation of PBMC with EpiCor drastically reduced the Fucoidan-induced CXCR4 ($P < .005$) and CCR9 ($P < .0007$) expression on NK cells. Data are expressed as mean ± SD, n = 3, and are representative of 3 similar experiments performed on lymphocytes from different donors.

expression in a significant manner for both CXCR4 ($P < .005$) and CCR9 ($P < .0007$) (Fig. 6B). This supports the data indicating a possible role of L-selectin in the EpiCor-mediated modulation of cellular responses to chemotactic signals during lymphocyte trafficking and migration.

4. Discussion

Chronic inflammation has grown to endemic proportions in industrialized countries, in correlation with a fast-paced lifestyle and diet [19-22]. An unbalanced immune activation, in response to pathogens from the gut lumen, has been implicated as a risk factor for many chronic inflammatory conditions, including irritable bowel disease, ulcerative colitis, and Crohn disease, and has been a contributing factor in obesity, heart disease, Alzheimer disease, and autoimmune diseases such as rheumatoid arthritis and psoriasis. Furthermore, chronic inflammation facilitates carcinogenesis and tumor progression [23,24]. There is a growing interest in unique foods and supplements that combine vitamins, antioxidants, and immunomodulating compounds to use nutritional strategies to reduce inflammation and support various aspects of immune function [25,26].

EpiCor is a new *S. cerevisiae*–based immunogen product with a high content of metabolites, including antioxidant compounds resulting in a reported high ORAC value [1] and high ROS scavenging activity [2]. Data presented here show that EpiCor possesses significant antioxidant and antiinflammatory activity over a wide range of dilutions in a cell-based assay. EpiCor inhibited ROS formation, as induced by H_2O_2, in human PMN cells. As several ROS species play roles in cell signaling, it is likely that EpiCor is capable of reducing the background noise of chronic inflammation, thereby increasing the capacity for maintaining balanced immune responses.

Furthermore, our data on EpiCor strongly indicate that it contains compounds that directly provide signals for human lymphocyte subsets. The direct activation of NK cells and B cells is significant. In addition, the data points to modulation of cell signaling are reflected by changes in proliferative activity, cytokine production, and chemokine receptor expression. This suggests a change in lymphocyte responsiveness and may indicate a shift from cellular (Th1) toward humoral (Th2) immune response in the presence of EpiCor. Of particular interest is the strong inhibition of IL-2–induced IFN-γ production. Interferon gamma is unique among the interferons and is the principal molecule for activation of macrophages. Because IFN-γ is produced by activated T-lymphocytes as part of an immune response, this supports the notion that EpiCor may favor Th2 responsiveness while simultaneously producing a strong activation of B cells and NK cells. It may also play a role in the physiology of chronic inflammatory conditions by reducing inflammation while optimizing the humoral response to pathogens.

Realistically, in a given person at a given time, Th1-type and Th2-type reactions are happening simultaneously in different microanatomical locations throughout the body. The spatial and temporal distance allows for fine-tuning of the overall immune defense. However, under chronic inflammatory conditions, the failure to balance Th1 and Th2 responses, possibly due in part to increased levels of inflammatory mediators, may contribute to a reduced capacity for optimal immune response to various pathogens.

Given the strong inhibition of chemokine receptor expression seen on human lymphocyte subsets after exposure to EpiCor in vitro, and based on EpiCor as a consumable product, a major antiinflammatory impact might be seen in the gut. Effects could hypothetically include modulation of trafficking of specific immune cell types and/or subsets. Chemokine antagonists are intensively researched as potent and highly selective pharmaceutical antiviral and antiinflammatory compounds [27-29]. Particularly, CCR9 plays a key role in homing to the gut a subset of $CD4^+$ T cells [30] and plasma cells [31]. Also, the homing of T cells to the liver in chronic liver disease is mediated by CCR9 [32].

Based on these in vitro studies, EpiCor has the potential to act as an immune modulator while at the same time helping to reduce gastrointestinal and, possibly, systemic inflammation. The data presented here on EpiCor can be seen as support for the benefit of further clinical work to determine the possible effect of EpiCor consumption on chronic inflammatory disorders, as well as viral diseases.

Acknowledgment

The authors thank Lue Ann Zaske, Kelly M. Patterson, Anna Gawlicka, John R. Endres, and Donna Kachinskas for their excellent technical assistance. The study was sponsored by Embria Health Sciences, Cedar Rapids, Iowa.

References

[1] Schauss AG. Suplemento alimentar unico que proporciona a melhoria imunologica. A novel yeast fermentate. Rev Bioquimica Med 2006;15(3):30-2.
[2] Schauss AG, Vojdani A. A discovery of an edible fermentation product with unusual immune enhancing properties in humans. FASEB J 2006;20:A143.
[3] Plat J, Mensink RP. Food components and immune function. Curr Opin Lipidol 2005;16:31-7.
[4] Kournikakis B, Mandeville R, Brousseau P, Ostroff G. Anthrax-protective effects of yeast beta 1,3 glucans. Med Gen Med 2003;5:1.
[5] Hong F, Hansen RD, Yan J, Allendorf DJ, Baran JT, Ostroff GR, et al. Beta-glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumoricidal granulocytes as killer cells. Cancer Res 2003;63:9023-31.
[6] Hong F, Yan J, Baran JT, Allendorf DJ, Hansen RD, Ostroff GR, et al. Mechanism by which orally administered beta-1,3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models. J Immunol 2004;173:797-806.

[7] Pelizon AC, Kaneno R, Soares AM, Meira DA, Sartori A. Down-modulation of lymphoproliferation and interferon-gamma production by beta-glucan derived from *Saccharomyces cerevisiae*. Mem Inst Oswaldo Cruz 2003;98:1083-7.
[8] Davis ME, Maxwell CV, Erf GF, Brown DC, Wistuba TJ. Dietary supplementation with phosphorylated mannans improves growth response and modulates immune function of weanling pigs. J Anim Sci 2004;82:1882-91.
[9] Cassone A, Marconi P, Bistoni F. Cell wall of *Candida albicans* and host response. Crit Rev Microbiol 1987;15:87-95.
[10] Roeder A, Kirschning CJ, Rupec RA, Schaller M, Weindl G, Korting HC. Toll-like receptors as key mediators in innate antifungal immunity. Med Mycol 2004;42:485-98.
[11] Boyum A. Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 1968;21(Suppl 97):77.
[12] English D, Andersen BR. Single-step separation of red blood cells. Granulocytes and mononuclear leukocytes on discontinuous density gradient of Ficoll-Hypaque. J Immunol Methods 1974;5:249.
[13] Schauss AG, Wu X, Prior RL, Ou B, Huang D, Owens J, et al. Antioxidant capacity and other bioactivities of the freeze-dried Amazonian palm berry, Euterpe oleraceae mart. (acai). J Agric Food Chem 2006;54(22):8604-10.
[14] Peters CE, Woodside SM, Eaves AC. Isolation of subsets of immune cells. Methods Mol Biol 2005;302:95-116.
[15] Horan PK, Slezak SE. Stable cell membrane labelling. Nature 1989;340(6229):167-8.
[16] Ding Z, Issekutz TB, Downey GP, Waddell TK. L-Selectin stimulation enhances functional expression of surface CXCR4 in lymphocytes: implications for cellular activation during adhesion and migration. Blood 2003;101(11):4245-52.
[17] Ludbrook J. Issues in biomedical statistics: comparing means under normal distribution theory. Aust N Z J Surg 1995;65(4):267-72.
[18] Livingston EH. Who was Student and why do we care so much about his *t*-test? J Surg Res 2004;118(1):58-65.
[19] Kontogianni MD, Zampelas A, Tsigos C. Nutrition and inflammatory load. Ann N Y Acad Sci 2006;1083:214-38.
[20] Hu FB, Willett WC. Optimal diets for prevention of coronary heart disease. JAMA 2002;288(20):2569-78.
[21] Fujioka K. Metabolic syndrome treatment strategies. Pharmacotherapy 2006;26(12 Pt 2):222S-6S.
[22] Kao PC, Shiesh SC, Wu TJ. Serum C-reactive protein as a marker for wellness assessment. Ann Clin Lab Sci 2006;36(2):163-9.
[23] Trosko JE. Dietary modulation of the multistage, multimechanisms of human carcinogenesis: effects on initiated stem cells and cell-cell communication. Nutr Cancer 2006;54(1):102-10.
[24] Schottenfeld D, Beebe-Dimmer J. Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. CA Cancer J Clin 2006;56(2):69-83.
[25] Tapsell LC, Hemphill I, Cobiac L, Patch CS, Sullivan DR, Fenech M, et al. Health benefits of herbs and spices: the past, the present, the future. Med J Aust 2006;185(4 Suppl):S4-S24.
[26] Philpott M, Ferguson LR. Immunonutrition and cancer. Mutat Res 2004;551(1-2):29-42.
[27] Barbaro G, Scozzafava A, Mastrolorenzo A, Supuran CT. Highly active antiretroviral therapy: current state of the art, new agents and their pharmacological interactions useful for improving therapeutic outcome. Curr Pharm Design 2005;11:1805-43.
[28] Li JH, Zhang J. PARP inhibitors. IDrugs 2001;4:804-12.
[29] Santoro F, Vassena L, Lusso P. Chemokine receptors as new molecular targets for antiviral therapy. New Microbiol 2004;27:17-29.
[30] Stenstad H, Ericsson A, Johansson-Lindbom B, Svensson M, Marsal J, Mack M, et al. Gut-associated lymphoid tissue–primed CD4+ T cells display CCR9-dependent and -independent homing to the small intestine. Blood 2006;107:3447-54.
[31] Pabst O, Ohl L, Wendland M, Wurbel MA, Kremmer E, Malissen B, et al. Chemokine receptor CCR9 contributes to the localization of plasma cells to the small intestine. J Exp Med 2004;199:411-6.
[32] Eksteen B, Grant AJ, Miles A, Curbishley SM, Lalor PF, Hubscher SG, et al. Hepatic endothelial CCL25 mediates the recruitment of CCR9+ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. J Exp Med 2004;200:1511-7.