In vitro human lymphocyte proliferative responses to a glycoprotein of the yeast Saccharomyces cerevisiae

C. J. DARROCH, S. E. CHRISTMAS & R. M. R. BARNES *Department of Immunology, University of Liverpool*

SUMMARY

Following reports of enhanced humoral immunity to *Saccharomyces cerevisiae* in patients with Crohn's disease, and identification of an immunodominant, high molecular weight glycoprotein (gp200), we have investigated the cellular immune response to this yeast in normal individuals. Following exposure to a crude saline extract (Sacc), peripheral blood mononuclear cells (PBMC) from these subjects demonstrated dose-dependent increases in tritiated thymidine incorporation, the time–course of which resembled that of the response to the known recall antigens PPD and TT. This was accompanied by increased cytotoxicity of the cultured cells for natural killer (NK)-sensitive and NK-resistant target cell lines. Furthermore, using a purified, high molecular weight, glycoprotein fraction of Sacc in culture, a dose-dependent lymphoproliferative response was again observed. Stimulation indices (SI) for thymidine incorporation by umbilical cord blood lymphocytes exposed to Sacc were low compared with those of normal adults. These results provide evidence for possible antigen-specific, cellular, immune sensitization of normal individuals to a ubiquitous dietary component.

INTRODUCTION

Recently, Main *et al.* using the enzyme-linked immunosorbent assay (ELISA) technique with a crude saline extract of *Saccharomyces cerevisiae* as solid phase antigen, reported the presence of specific IgG and IgA antibodies in the serum of patients with Crohn's disease, and which distinguished this group from normal controls and patients with ulcerative colitis.[1] In the case of antibodies of the IgG isotype, this observation held true for 11 of 12 *S. cerevisiae* strains examined, and there was no serological cross-reactivity with two strains of *Candida albicans*.[2] Results from this laboratory confirmed and extended these findings, suggesting that IgA anti-Sacc antibody, though less sensitive, was a more specific marker for Crohn's disease;[3] furthermore, there was no correlation between the presence of anti-Sacc antibodies and those reacting with *Escherichia coli* or other dietary antigens. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by periodate oxidation and immunoblotting with antibody-positive sera suggest that the relevant antigen is a high molecular weight (~ 200,000 MW), heat-stable, soluble glycoprotein (gp200).[4]

The demonstration of specific humoral immunity to this putatively non-pathogenic organism, quantitatively different in subjects with a specific pathology as compared with normal individuals, raises the possibility that concomitant cellular immunity to the same antigen is also present. The fact that *in vitro* immune responses to the pathogenic fungus *C. albicans*

have been extensively studied[5][11] has provided a model for the study of this related organism and we have previously reported preliminary findings of increased *in vitro* DNA synthesis by peripheral blood mononuclear cells (PBMC) in response to a crude saline extract of the yeast (Sacc), which suggest that this is the case even in normal subjects.[12,13]

The aim of the present work has been to confirm and extend these observations by making a qualitative comparison between lymphocyte proliferative responses to Sacc and those to recognized recall antigens and mitogens, thereby establishing optimal culture conditions with which to explore the phenomenon of cell-mediated cytotoxicity induced by Sacc. The lymphoproliferative response to the previously identified high molecular fraction of Sacc has also been examined.

MATERIALS AND METHODS

Preparation of cells

PBMC were obtained from normal subjects by the method of Böyum.[14] Briefly, aliquots of whole blood were collected from donors by venepuncture and diluted with sterile, heparinized (15 U/ml final concentration) physiological saline (ratio of blood : saline = 1 : 1 to 2 : 1). This mixture was then separated on a Lymphoprep (Nycomed, Oslo, Norway) density gradient by centrifugation at 2000 rpm (~ 830 *g*) for 20 min at 20°. The buffy layer was removed by pipette and the cells washed once in an excess of wash fluid [RPMI-1640/25 mM HEPES (Flow Laboratories, Irvine, U.K.) plus heparin at 15 U/ml)] followed by centrifugation at 1400 rpm (~ 400 *g*) for 15 min at 20°. After discarding the supernatant, the pellets were resuspended in a small volume of complete culture medium [RPMI/HEPES, each

100 ml supplemented with the following: 3 ml 7·5% NaHCO$_3$ solution (Gibco, Paisley, U.K.); 10 ml heat-inactivated (56° for 30 min), sterile-filtered (0·2 μm), human AB serum; 2 ml L-glutamine 200 mM (Gibco) and 2 ml of a solution containing penicillin 5000 U/ml plus streptomycin 5000 μg/ml (Gibco)] prior to counting. Cells obtained by this method were virtually 100% viable by trypan blue exclusion.

Heparinized umbilical cord blood was first depleted of erythrocytes by preincubating each 10 ml aliquot with 2 ml 1% methylcellulose (Sigma, St Louis) in phosphate-buffered saline (PBS) at 37° for 15 min with mixing, then diluting with an equal volume of physiological saline and allowing to settle for a further 30 min prior to density gradient centrifugation of the supernatant suspension with Lymphoprep.

Antigens and mitogens

Sacc. A saline extract of *S. cerevisiae* was prepared as previously described.[4] Briefly, 100 g of a commercially available dried bakers' yeast (Sainsbury's) was suspended in 500 ml sterile physiological saline at 4° and washed twice, resuspending to the original volume after each centrifugation. Following the second wash, the suspension was heated in a water-bath at 100° for 1 hr. After cooling, the suspension was centrifuged, and the supernatant sterile filtered to 0·2 μm. Total protein concentration was measured by the Coomassie brilliant blue colorimetric method (Bio-Rad, Richmond, CA), and the preparation was stored in aliquots at −20° until further use.

A purified high molecular weight fraction was prepared by subjecting an aqueous extract (as above, but prepared in water rather than saline) to an approximately sixfold volume reduction by ultrafiltration using a TCF 2-micro-thin channel ultrafiltration system (Amicon, Lexington, MA) with membranes having a 50,000 MW cut-off; the ultrafiltrate was then lyophilized and that product was then applied to a Sepharose CL6B column (Pharmacia, Uppsala, Sweden) equilibrated in sterile water, and the fraction eluting in the void volume was collected and lyophilized. This fraction showed a single band of apparent MW = 200,000 after SDS–PAGE and staining with Coomassie brilliant blue and periodic acid Schiff's reagent (PAS).

Purified protein derivative of Mycobacterium tuberculosis *(PPD).* A pharmaceutical preparation containing 100,000 U/ml PPD (Evans Medical, Horsham, U.K.) was obtained. This had a total protein concentration of 750 μg/ml and was stored until further use at 4°, according to the manufacturer's recommendations.

Tetanus toxoid (TT). Unadsorbed tetanus vaccine, 60 ml (Evans Medical) was dialysed extensively against distilled water, sterile filtered (0·2 μm), and lyophilized; it was then redissolved to 10 ml in water, centrifuged, refiltered and stored at −20° until further use.

Pokeweed mitogen (PWM). A stock solution was prepared by dissolving 10 mg lyophilized mitogen (Sigma) in 5 ml sterile water. Aliquots were stored at −20° until further use.

Interleukin-2 (IL-2). A stock solution of purified lymphoblastoid IL-2 (Biotest, Dreieich, Germany) containing 100,000 U/ml was stored at 4°.

Proliferation assays

For dose–response and time–course experiments, PBMC were adjusted to 2/3 × 10^6 lymphocytes/ml in culture medium and plated on to sterile 96-well, U-bottomed culture plates (Flow) at 10^5 cells in 150 μl/well. Dilutions of antigens/mitogens were prepared in culture medium at four times their intended final concentrations in culture and added to the wells in 50 μl volumes; the same volume of culture medium alone was added to control wells. Plates were then incubated for variable periods at 37° in a humid atmosphere containing 5% CO$_2$.

On the day of harvesting, the cells were pulsed with [methyl-^{3}H]thymidine (1 mCi/ml) (Amersham International, Amersham, U.K.) by adding 1 μl to each well and incubating for a further 6 hr. The cells were then harvested on to glass fibre filters using a semi-automatic cell harvester (Flow). The filters were allowed to dry overnight prior to β-counting of the individual discs in "OptiScint 'Hi Safe'" liquid scintillant (LKB, Surrey, U.K.).

Medians were calculated for each group of replicate wells. Results are expressed either as absolute c.p.m. or as the stimulation index (SI: count for stimulated culture/count for corresponding unstimulated control culture).

Cytotoxicity assay

Effector cells. PBMC were cultured in bulk at 5 × 10^5 lymphocytes/ml in 50-ml flasks and under the same incubation conditions as described above for the proliferative assay. Four cultures were established for each donor, as follows: unstimulated cells; cells plus Sacc; cells plus PPD; cells plus IL-2. After 7 days, the cells were washed in RPMI supplemented with 10% fetal calf serum (FCS), resuspended in a small volume of the medium, and a viable count (trypan blue exclusion) performed. Finally, the cells were adjusted to 5 × 10^5/ml.

Target cells. Two cell lines were used: mel-1, a natural killer (NK)-resistant, malignant melanoma-derived cell; and Molt4, an NK-sensitive, T-cell leukaemia-derived cell. These were harvested from culture, washed twice in RPMI/FCS and the centrifuged pellets were resuspended in the presence of 10 μl of a solution of ^{51}Cr-sodium chromate (10 mCi/ml) (Flow). After incubation for 1 hr at 37°, the cells were washed twice in RPMI/FCS and adjusted to 5 × 10^4/ml after performing a viability count.

The assay was performed by adding 150 μl of each effector cell suspension to the same volume of each target cell suspension in 11-mm diameter test-tubes in triplicate, to give an effector to target cell ratio of 10:1. After gentle centrifugation at 1,000 rpm (∼208 g) for 5 min, followed by incubation for 4 hr at 37°, 150 μl of supernatant was removed from each tube. Respective pairs of pellets and supernatants were then counted in a γ-counter. Spontaneous release was calculated from tubes containing target cells plus 150 μl medium only, and maximum release from tubes containing target cells plus 150 μl 2% Triton 100 (BDH) to lyse the cells. A mean background count was subtracted from each experimental count, and the per cent cytotoxicity for each pair of tubes calculated as follows:

$$\% \text{ cytotoxicity} = \frac{R_{\text{test}} - R_{\text{spont}}}{R_{\text{max}} - R_{\text{spont}}} \times 100$$

where: R_{test} = fractional release in test sample; R_{spont} = mean spontaneous fractional release; R_{max} = mean maximum fractional release. The mean of each triplicate was taken as the final result.
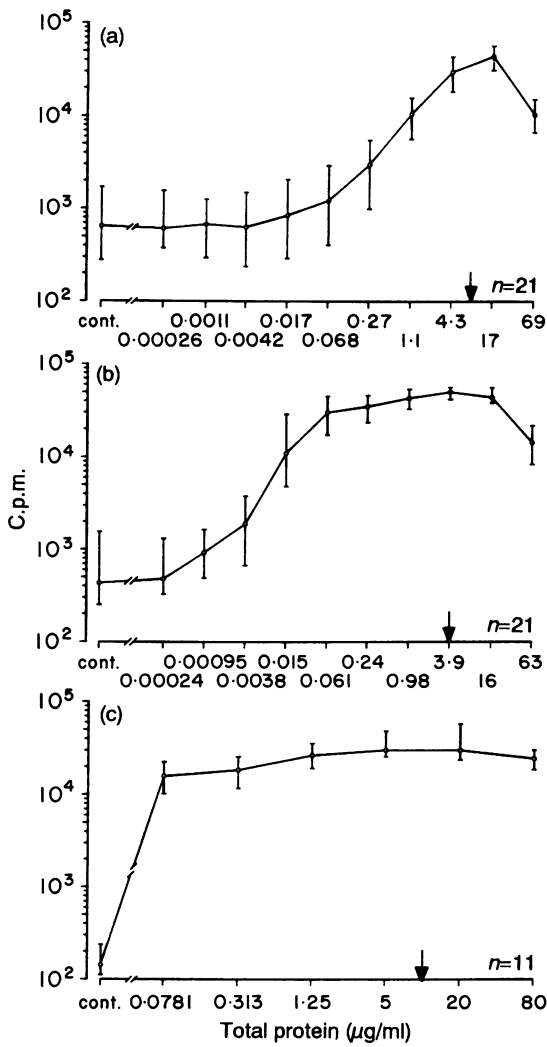
Figure 1. Dose–response relationship for: (a) Sacc; (b) PPD; (c) PWM. In (a) and (b), eight replicate wells were used for each subject at each dose, and six replicates in (c); the final response for each group of replicates being the median of the individual counts. These medians were then used to estimate the population median and the 95% confidence interval (Wilcoxon) at that dose. Figures on the horizontal axes refer to final concentrations in culture; cont. = control, medium only added to culture. Arrows indicate concentrations used for the subsequent time–course experiment.
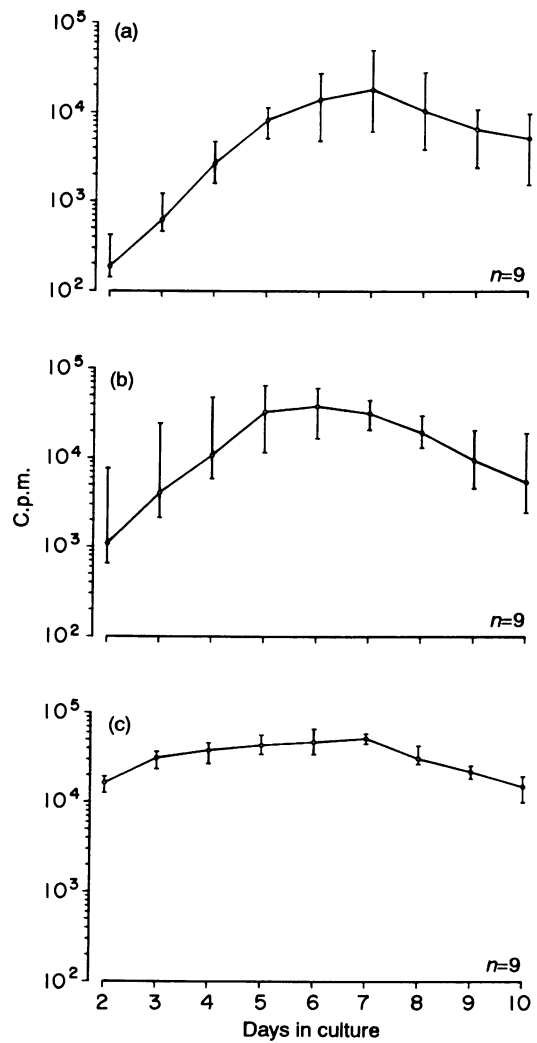
Figure 2. Time–course of response to: (a) Sacc (8·6 μg/ml); (b) PPD (3·9 μg/ml); (c) PWM (10 μg/ml). Each experiment was performed on six replicate wells and the analysis was as for the data in Fig. 1.

Statistical analysis

Data were compared using the appropriate non-parametric tests in MINITAB (MINITAB, State College, PA), for personal computers.
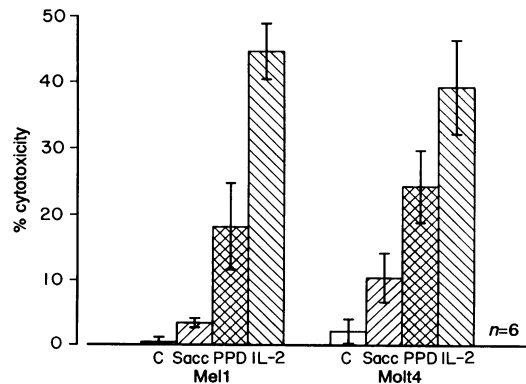
RESULTS

### Dose–response and time–course

After 7 days in culture, the proliferation, as measured by tritiated thymidine incorporation over 6 hr, of PBMC from normal donors in response to a range of fourfold dilutions of Sacc, PPD and PWM is shown in Fig. 1, where the median and 95% confidence interval for the sample is plotted at each concentration. A major feature is that the demonstration of an optimal response to Sacc appears to be more critically depen-
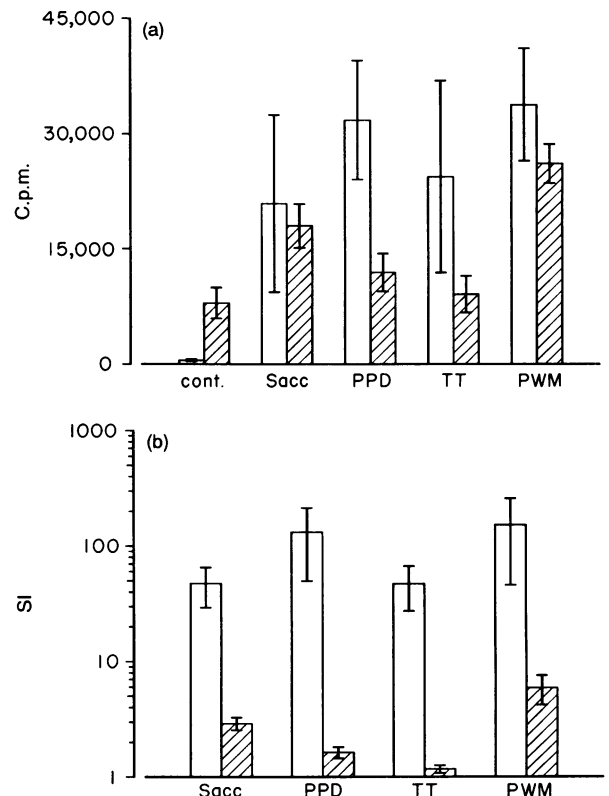

Figure 3. Cytotoxicity assay. Effector cells were obtained by culture of lymphocytes from six normal donors: C = control (unstimulated) cells. Concentrations of Sacc and PPD used for preculture of effector cells were as for Fig. 2; IL-2 was used at 50 U/ml. Bars show mean $\pm$ SEM % cytotoxicity by effector cells, incubated in a ratio of 10:1 with target cells.
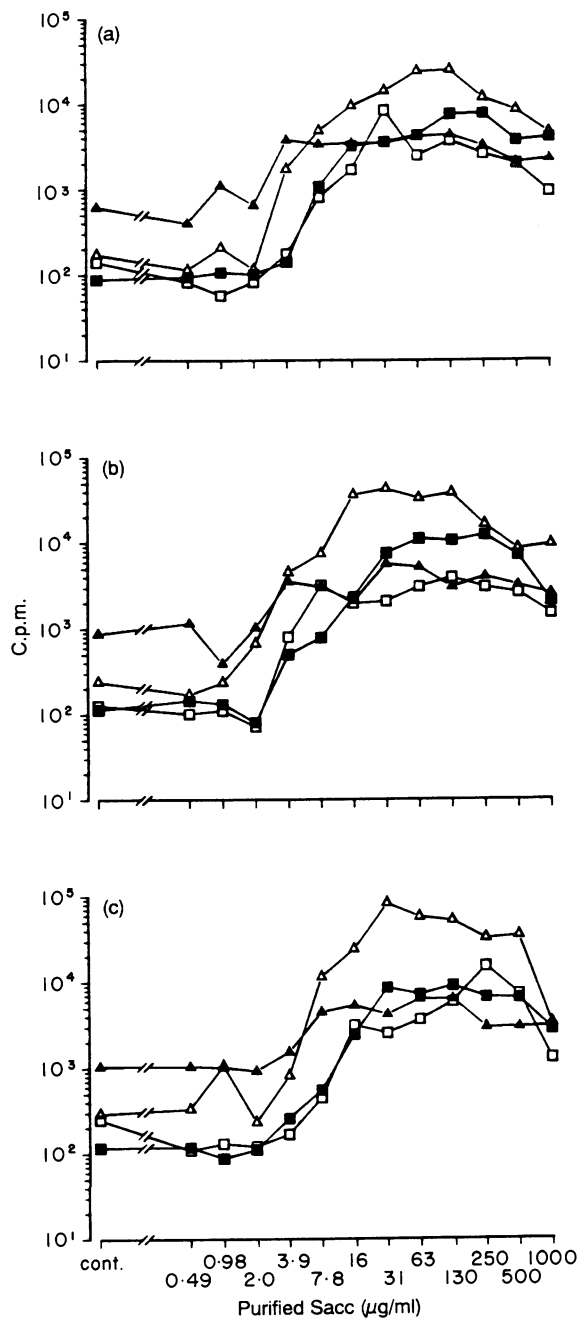
Figure 4. Proliferative response to purified Sacc. Cells were harvested on 3 consecutive days: (a) day 6; (b) day 7; (c) day 8. Each data point is the median of five replicates. Separate dose–response curves are shown for each of the four subjects.

Figure 5. Comparison of proliferative responses of PBMC (□, $n=4$) and CBMC (■, $n=9$), expressed as: (a) absolute c.p.m.; (b) SI. Bars show mean ± SEM derived from five replicates.

responses to Sacc and PPD were delayed at 7 days and 5–6 days respectively.

The dose dependency and time–course of the response to TT (not shown) closely resembled that of PPD.

Cytotoxicity

The per cent cytotoxicity obtained against the mel-1 and Molt4 cell lines with an effector to target cell ratio of 10:1 is shown in Fig. 3. Similar results were obtained for ratios of 20:1 and 5:1 (data not shown). All effector cell samples demonstrated cytotoxicity significantly greater than that of the unstimulated control at $P<0.05$ (Wilcoxon signed rank test). Antigen-stimulated cells elicited greater killing against the NK-sensitive line (Molt4) compared with the NK-resistant line (mel-1).

Proliferative responses to purified Sacc

PBMC were obtained from four donors who had previously demonstrated significant proliferative responses to Sacc and tested for their ability to proliferate in response to doubling dilutions of a high molecular weight fraction purified by gel filtration. Figure 4 shows that the responses were comparable regardless of whether cells were harvested after 6, 7 or 8 days in culture. Furthermore, it can be seen that the amount of thymidine incorporation is less critically dependent on dose than was the case for the unfractionated Sacc preparation, and in this respect the response more closely resembles those of PPD and TT.

dent on dose than is the case for PPD and PWM, which both produce a shallow plateau of responses over a greater than 200-fold concentration range.

Data on the time–course of the proliferative responses (Fig. 2) were obtained by measuring tritiated thymidine incorporation daily in the presence of that concentration of Sacc, PPD and PWM which had produced the optimal response at 7 days (these are indicated by arrows against the horizontal axes in Fig. 1). Whereas PWM elicited very early proliferation which was maintained throughout the course of the experiment, peak

Proliferative response of cord blood lymphocytes

Cord blood mononuclear cells (CBMC) were incubated with concentrations of Sacc, PPD, TT and PWM which had been shown to be optimal for adult PBMC. After 7 days in culture, the incorporation of tritiated thymidine was compared with that of similar cultures of normal adult PBMC (Fig. 5). Although the responses, in absolute c.p.m. of stimulated CBMC cultures were in each case not significantly different from those of PBMC (Fig. 5a), unstimulated CBMC cultures uniformly demonstrated a high background uptake [$P < 0.01$ (Mann–Whitney) cf. PBMC] with consequently reduced SI for stimulated cultures [$P < 0.01$ (Mann–Whitney) cf. PBMC] (Fig. 5b).

DISCUSSION

Saccharomyces cerevisiae is not recognized as a pathogen except in rare, anecdotal cases. However, exposure to this organism in the form of bakers'/brewers' yeast is virtually universal and the demonstration of humoral immunity to the yeast extract Sacc, specifically in relation to Crohn's disease,[1-4] raises the possibility of sensitization via the gut-associated lymphoid tissue (GALT), a site at which activated T lymphocytes may be involved in induction or maintainance of gastrointestinal disease.[15]

Using incorporation of tritiated thymidine as a marker of DNA synthesis in PBMC, it has been possible to establish dose–response relationships for Sacc, PPD and PWM after 7 days in culture (Fig. 1). Although the profile of the Sacc response appears to differ markedly from those of PPD and PWM, in so far as optimal thymidine uptake is more critically dependent on a narrow range of concentrations, this may have been an artefact due to the nature of the preparation used: that is, above a concentration of total Sacc protein of 17 μg/ml it may not have been possible to increase the proportion of the saline extract in the culture medium without either introducing significant amounts of inhibitory factors or excessively diluting essential components of the medium. Indeed, this anomaly was not observed with the purified, lyophilized, high molecular fraction (see below).

Having thus established appropriate optimal concentrations, the time–course of each response was examined (Fig. 2). The kinetics of the responses to Sacc and PPD are similar and contrast with that to PWM in that the maximum responses are delayed, reaching well-defined peaks on day 7 (Sacc) and day 6 (PPD), compared with the early plateau of thymidine uptake seen with PWM. It could be argued that this observation supports the suggestion that Sacc, like PPD, is mediating lymphocyte activation in an antigen-dependent manner rather than acting non-specifically as a mitogen. This conclusion might be criticized on the grounds that a maximal response at about 7 days has already been preselected on the basis of the doses chosen and that the earlier response to PWM merely reflects less stringent dose requirements rather than a fundamental difference in the kinetics of the response *per se*. However, when tetanus toxoid was used as recall antigen, it also resulted in maximum uptake at 5–7 days, and this remained true over a greater than 200-fold dose range (results not shown). The kinetics of the response to Sacc are in broad agreement with those reported elsewhere for antigenic preparations of *C. albicans*.[5,6]

In previous studies in mouse and man, *C. albicans*-stimulated cells have been shown to possess *in vitro* cytotoxic activity

against a variety of allogeneic targets and this has been attributed to induction of NK cell activity.[7-9] Sacc also appears to have this property: the cytotoxicity of Sacc-stimulated cells for both NK-sensitive and NK-resistant cell lines was statistically greater than that demonstrated by negative control cells (Fig. 3). The levels of cytotoxic activity induced by Sacc were less than those obtained with the control antigen PPD, and this may reflect differences in the precursor frequency or phenotype of lymphocyte subsets which each activates. In the studies on *C. albicans* cited, cytotoxicity was also greater, and although the different target cell lines, and in some cases, higher effector:target cell ratios used in those experiments make direct comparison with the current data difficult, Sacc does appear to be a less potent inducer of cytotoxicity than *C. albicans*.

The ability of a purified high molecular weight glycoprotein fraction of Sacc to elicit a lymphoproliferative response was examined, and, as is shown in Fig. 4, this retained similar efficacy to the crude preparation. This fraction had the electrophoretic and staining characteristics of the previously identified gp200,[4] properties also shared by a heat-stable mannoprotein fraction of *C. albicans* which was recognized by anti-*Candida* rabbit serum and has been demonstrated to induce lymphoproliferation and cytotoxicity *in vitro*.[9]

Umbilical cord blood contains T cells which are said to be phenotypically and functionally immature.[16] In some studies, *in vitro* CBMC responsiveness to a stimulating substance, for example, mannan of *C. albicans*,[17] has been taken to imply mitogenicity. However, weak CBMC responses to antigenic stimuli are detectable, and, in the case of food antigens, have been proposed as predictors of future allergy.[18] In addition, limiting dilution analysis has demonstrated a precursor frequency of PPD-specific cells in cord blood only 10- to 100-fold less than adult peripheral blood, and no difference in the frequencies of cells from these populations which respond to the 65,000 MW mycobacterial heat-shock protein.[19] Because cell preparations may differ in their spontaneous rate of thymidine uptake, the SI is often used as a device to normalize data from lymphoproliferation assays; we, and others,[16] have found CBMC to generate particularly high background counts. Although results manipulated in this way should be interpreted with caution, our experiments showed CBMC responses to Sacc, PPD and TT which, expressed as SI, were significantly less than those of PBMC, whereas there was no difference between cell populations when results were expressed in c.p.m. Microscopic visual inspection of the cultures tended to support the conclusion that significant proliferation of CBMC only occurred in response to PWM; in this latter instance, the apparently low SI may have been due to the fact that it was measured after the peak response had been achieved.

In conclusion, in keeping with the apparent ability of *S. cerevisiae* to elicit humoral immune responses in man *in vivo*, soluble preparations of the organism can induce a lymphoproliferative response *in vitro* which is kinetically similar to that due to known recall antigens. This property is retained by a high molecular weight fraction and is accompanied by the ability to induce cytotoxic activity in stimulated cells. Whether the response of PBMC to Sacc is truly antigen specific remains to be established, as the evidence presented here, based on the kinetics of the response and hyporesponsiveness of CBMC, is merely suggestive. However, it has been shown (C. J. Darroch, S. E. Christmas and R. M. R. Barnes, unpublished observations) that

randomly selected T-cell clones, which proliferate in response to PWM and phytohaemmagglutinin, are not stimulated by Sacc, and work is in progress to attempt to isolate clones which are specifically responsive to Sacc.

Although the biochemical nature of the active component(s) of Sacc has not been fully characterized, the method of preparation, possession of electrophoretic and staining characteristics of a high molecular weight glycoprotein, and the known immunological properties of yeasts[10,11] make cell wall mannan the most likely candidate (preliminary analysis of the purified preparation supports this suggestion).

It is not known what relevance the ubiquitous presence of *S. cerevisiae* in food has to these observations. Lymphoproliferative responses to other food antigens are not universally demonstrable in normal individuals;[20] Sacc may be unusual in this respect, and further study is necessary to characterize its role in health and disease.

REFERENCES

1. MAIN J., McKENZIE H., YEAMAN G.R., KERR M.A., ROBSON D., PENNINGTON C.R. & PARRATT D. (1988) Antibody to *Saccharomyces cerevisiae* (bakers' yeast) in Crohn's disease. *Br. Med. J.* **297**, 1105.

2. McKENZIE H., MAIN J., PENNINGTON C.R. & PARRATT D. (1990) Antibody to selected strains of *Saccharomyces cerevisiae* (baker's and brewer's yeast) and *Candida albicans* in Crohn's disease. *Gut*, **31**, 536.

3. BARNES R.M.R., ALLAN S., TAYLOR-ROBINSON C.H., FINN R. & JOHNSON P.M. (1990) Serum antibodies reactive with *Saccharomyces cerevisiae* in inflammatory bowel disease: is IgA antibody a marker for Crohn's disease? *Int. Archs Allergy Appl. Immunol.* **92**, 9.

4. HEELAN B.T., ALLAN S. & BARNES R.M.R. (1991) Identification of a 200-kDa glycoprotein antigen of *Saccharomyces cerevisiae*. *Immunol. Lett* **28**, 181.

5. PICCOLELLA E., LOMBARDI G. & MORELLI R. (1980) Human lymphocyte-activating properties of a purified polysaccharide from *Candida albicans*: B and T cell cooperation in the mitogenic response. *J. Immunol.* **125**, 2082.

6. AUSIELLO C.M., SPAGNOLI G.C., BOCCANERA M., CASALINUOVO I., MALAVASI F., CASCIANI C.U. & CASSONE A. (1986) Proliferation of human peripheral blood mononuclear cells induced by *Candida albicans* and its cell wall fractions. *J. Med. Microbiol.* **22**, 195.

7. SCARINGI L., MARCONI P., BOCCANERA M., TISSI L., BISTONI F. & CASSONE A. (1988) Cell wall components of *Candida albicans* as immunomodulators: induction of natural killer and macrophage-mediated peritoneal cell cytotoxicity in mice by mannoprotein and glucan fractions. *J. gen. Microbiol.* **134**, 1265.

8. AUSIELLO C.M., PALMA C., SPAGNOLI G.C., PIAZZA A., CASCIANI C.U. & CASSONE A. (1989) Cytotoxic effectors in human peripheral blood mononuclear cells induced by a mannoprotein complex of *Candida albicans*: a comparison with interleukin 2-activated killer cells. *Cell. Immunol.* **121**, 349.

9. TOROSANTUCCI A., PALMA C., BOCCANERA M., AUSIELLO C.M., SPAGNOLI G.C. & CASSONE A. (1990) Lymphoproliferative and cytotoxic responses of human peripheral blood mononuclear cells to mannoprotein constituents of *Candida albicans*. *J. gen. Microbiol.* **136**, 2155.

10. NELSON R.D., SHIBATA N., PODZORSKI R.P. & HERRON M.J. (1991) *Candida* mannan: chemistry, suppression of cell-mediated immunity, and possible mechanisms of action. *Clin. Microbiol. Rev.* **4**, 10.

11. REES E. (1986) *Molecular Immunology of Mycotic and Actinmycotic Infections*, edn 1. Elsevier Science, New York.

12. BARNES R.M.R., ALLAN S., HEELAN B.T. & DARROCH C.J. (1991) Immune responses to *Saccharomyces cerevisiae* in Crohn's disease. *Eur. J. Gastroenterol. Hepatol.* **3** (suppl. 1), S59.

13. BARNES R.M.R. & DARROCH C.J. (1992) *In vitro* cellular responses to *Saccharomyces cerevisiae* in man. *Allergy*, **47**(suppl.), 321.

14. BÖYUM A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. clin Lab. Invest.* **21**(suppl. 97), 77.

15. MacDONALD T.T. (1990) The role of activated T lymphocytes in gastrointestinal disease. *Clin. exp. Allergy*, **20**, 247.

16. HARRIS D.T., SCHUMACHER M.J., LOCASCIO J., BESENCON F.J., OLSON G.B., DE LUCA D., SHENKER L., BARD J. & BOYSE E.A. (1992) Phenotypic and functional immaturity of human umbilical cord T lymphocytes. *Proc. natl. Acad. Sci. U.S.A.* **89**, 10006.

17. TOLLEMAR J., RINGDÉN O. & HOLMBERG K. (1989) *Candida albicans*: Mannan and protein activation of cells from various human lymphoid organs. *Scand. J. Immunol.* **30**, 473.

18. KONDO N., KOBAYASHI Y., SHINODA S., KASAHARA K., KAMEYAMA T., IWASA S. & ORII T. (1992) Cord blood lymphocyte responses to food antigens for the prediction of allergic disorders. *Arch. Dis. Child.* **67**, 1003.

19. FISCHER H.P., SHARROCK C.E. & PANAYI G.S. (1992) High frequency of cord blood lymphocytes against mycobacterial 65-kDa heat-shock protein. *Eur. J Immunol.* **22**, 1667.

20. FRIERI M., CLAUS M., BORIS M., ZITT M., SCALISE D. & HARRIS N. (1990) Preliminary investigation on humoral and cellular immune responses to selected food proteins in patients with Crohn's disease. *Ann. Allergy*, **64**, 345.