

Specific In Vitro Antimannan-Rich Antigen of *Candida albicans* Antibody Production by Sensitized Human Blood Lymphocytes

A. DURANDY, A. FISCHER, and C. GRISCELLI, *Groupe de Recherches d'Immunologie et de Rhumatologie Pédiatriques, Unité 132, Hôpital des Enfants Malades, Institut National de la Santé et de la Recherche Médicale, 75730 Paris Cédex 15, France*

ABSTRACT We have developed a new antigenic system for the induction of specific in vitro antibody response in man. The antigen used was purified from the cell wall of *Candida albicans* strain A and contained >96% polysaccharide mannan. Peripheral blood mononuclear cells from *Candida*-sensitized donors produced specific antimannan antibodies during a 7-d culture in the presence of mannan absorbed with methylated bovine serum albumin. Two methods were used to detect antimannan antibody responses. Antimannan antibody-producing cells were identified by radioautography with tritiated mannan. Antibody concentration in culture supernatants was measured by an enzyme-linked immunosorbent assay. In both methods, specific IgM and IgG (but not IgA) antibodies were detected. The antibody production to mannan was specific, since an antigenically unrelated polysaccharide (pneumococcal antigen S III) did not bind to methylated bovine serum albumin-mannan-induced blast cells and did not induce antimannan antibody-containing cells. Furthermore, a pulse with an excess of unlabeled mannan abolished [³H]mannan binding, whereas an excess of unlabeled S III did not. Similarly, no antimannan antibody was obtained in influenza virus-stimulated cultures and mannan-stimulated cultures were not inducing antiinfluenza antibodies. The antimannan antibody production was shown to be a T cell-dependent phenomenon. The T helper effect appeared to be radiosensitive. It was under a genetic restriction as it occurred only in autologous or semi-identical but not in allogeneic situations. This system is relatively simple, reproducible, and well suited for

the study of specific secondary in vitro antibody responses to polysaccharide antigens in humans.

INTRODUCTION

Most of the studies reported so far on the regulation of human B cell activation have been performed using polyclonal activation of B cells by mitogens (1, 2). Recently, however, several systems have been described for the generation of specific antibody production in vitro in primary responses to particulate antigens like sheep erythrocytes (3-5) and trinitrophenyl polyacrylamide beads (6) or to soluble antigens such as ovalbumin (3, 4). Secondary in vitro responses to influenza viruses (7, 8), tetanus toxoid (9), or keyhole limpet hemocyanin (10, 11) have also been described. We report here a new system of in vitro antibody production using an antigen prepared from *Candida albicans* cell wall and containing >96% pure mannan and that we called mannan. Blood lymphocytes from *Candida albicans* immune donor have been tested for in vitro antibody production. The assay involves identification of specific antibody-containing cells (ACC)¹ by radioautography with tritiated mannan together with measurement of antimannan antibody in culture supernatants using an enzyme-linked immunosorbent assay (ELISA). The system appears to be useful for the

¹ *Abbreviations used in this paper:* AC, adherent cells; ACC, antibody-containing cells; BSA, bovine serum albumin; E(+), cells that form spontaneous rosettes with neuraminidase-treated sheep erythrocytes; E(-), cells depleted of spontaneous rosettes with sheep erythrocytes; ELISA, enzyme-linked immunosorbent assay; ICC, immunoglobulin-containing cells; MBSA, methylated BSA; PBL, peripheral blood lymphocytes; PWM, pokeweed mitogen.

Received for publication 29 June 1982 and in revised form 5 January 1983.

analysis of the cellular requirements for antibody production in a secondary response to a polysaccharide antigen as well as for the study of the interaction between T, B lymphocytes, and monocytes in antibody production in man. It could also be used for the analysis of antibody production in patients with immune deficiency diseases, like the Wiskott-Aldrich syndrome, in which an impairment of antibody production to polysaccharides has been described (12) or the chronic candidiasis, in which high levels of antibody to *Candida* antigens contrast with an impaired specific cellular immunity (13, 14).

METHODS

Polysaccharide antigens. The antigen used was extracted from *Candida albicans* strain A, by a technic previously reported by Hasenclever (15). Briefly, the yeast cells were kept in an autoclave at 140°C for 2 h, in citrate buffer pH 7. Supernatants precipitated with ethanol were dissolved in water, and Fehling's solution was added. Mannan antigen was extracted by precipitation of copper insoluble complexes. Despite repeated purifications by this method, the acid hydrolysate still contained traces of glucose, as determined by paper chromatography. The protein contamination was 3.4% as determined by the Lowry method (16). Several peaks of mannan were found after gel filtration on an Ultrogel ACA 44 column (LKB Instruments, Inc., Stockholm, Sweden). The 8,000- and 15,000-mol wt forms were predominant. Carbohydrate concentration was determined by the phenyl sulfuric acid method (16). Since mannan was the main component of the antigen preparation (>96%), it was called mannan.

In some experiments, we used an unrelated polysaccharide as control, i.e., pneumococcal SIII polysaccharide (350,000 mol wt), prepared as originally described by Felton (16).

Mannan and SIII antigens were tritiated by L. Pichat (Centre d'Energie Atomique, France) by catalysed exchange in solution with tritium as described by Evans et al. (17). After labeling, both fractions of mannan (8,000 and 15,000 mol wt) were separated by ACA 44 gel filtration.

Analysis of [³H]mannan by acid hydrolysis and paper chromatography revealed that the whole radioactivity was bound to mannose. The Lowry determination gave evidence of a protein contamination (10.4%). Specific activity was 1.5 mCi/mg for [³H]mannan and 2 mCi/mg for [³H]SIII, respectively. Tritiated polysaccharides were filtrated on Millipore filter (Millipore Continental Water Systems, Bedford, MA) and stored in aqueous solution at +4°C. No spontaneous radiolysis was detectable since 3 yr after labeling of mannan nearly the whole radioactivity was still bound to mannose.

Absorption of mannan to methylated bovine serum albumin (MBSA—Sigma Chemical Co., St. Louis, MO) was performed at a ratio 4:1 as previously described for other polysaccharides by Wiener and Bandieri (18).

Cell separations. Blood samples were obtained from healthy adult volunteers or from cord blood on preservative-free heparin (100 IU/ml). Donors were tested for the presence of anti-*Candida* antibody in serum by electrosyneresis and/or by hemagglutination (19), and only seropositive donors were considered as sensitized. Some adult donors were immunised by pneumococcal SIII antigen, subcutaneously at a dose of 50 µg in 1 ml of saline, 15–30 d before being tested. Peripheral blood leukocytes (PBL) were isolated on

a Ficoll-Hypaque density gradient and washed twice in Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Glasgow, Scotland). B lymphocyte markers were determined by direct immunofluorescence using rhodamine-labeled goat F(ab')₂ fragments against human Fab' IgG or against human heavy chains (µ or γ) (Nordic Laboratories, Tilburg, The Netherlands). T lymphocytes were enumerated by using monoclonal mouse antibodies against all T lymphocytes (OKT3, Ortho Pharmaceutical, Raritan, NJ) revealed by a rhodamine-labeled goat anti-mouse Ig antiserum (Nordic Laboratories). Monocytes were identified either by indirect immunofluorescence (with monoclonal antibodies OKM1, Ortho Pharmaceutical) or by latex particles phagocytosis (20).

PBL preparations contained 15.8±8.2% monocytes, 6.1±3.5% B lymphocytes, and 78.2±10.1% T lymphocytes.

E(+) cells obtained after a 1-h incubation with neuraminidase (Behring Werke, Marburg-Lahn, Germany)-treated sheep erythrocytes were separated on a Ficoll-Hypaque gradient as previously described (21). This T cell-enriched population contained 87.9±6.8% OKT3 (+) cells, 0.5±0.4% slg (+) lymphocytes, and 2.1±0.8% monocytes. E(−) preparations were contaminated with few T cells (4.9±3.2%), which were eliminated by cytotoxicity with OKT3 in presence of rabbit complement (Institut Mérieux, Lyon, France). These T cell-depleted populations contained <1% OKT3 (+) cells, 47.3±7.5% slg (+) lymphocytes and 38.5±17.3% monocytes. In some experiments E(−) cell preparations were seeded three times in flat-bottom microwells (Falcon Plastics, Oxford, England) for 1 h at 37°C. Non-adherent E(−) cell populations contained 78.2±10.1% slg(+) lymphocytes and 20.0±18.1% monocytes. Adherent cells (AC) were shown to be mostly monocytes as judged by latex phagocytosis (≥90%). Around 20% of E(−) cells adhered to plastic.

In some experiments, cells were irradiated in a gamma-cell irradiator at doses ranging from 100 to 3,000 rad.

Cell cultures. PBL were cultured in flat- or round-bottom microtiter wells (Falcon Plastics) at 2 × 10⁵ in 0.2 ml RPMI 1640 (Gibco Laboratories) culture medium supplemented with 10% heat-inactivated fetal calf serum (Microbiological Associates, Bethesda, MD), antibiotics, (penicillin 100 IU/ml, streptomycin 0.1 mg/ml), amphotericin B (4 µg/ml), and glutamine (0.3 mg/ml, Institut Mérieux).

Cultures were stimulated by varying amounts of either mannan or mannan absorbed with MBSA (5–500 µg/ml). Some cultures were initiated with MBSA alone (1–100 µg/ml). In control experiments, two other antigens were used: pneumococcal polysaccharide SIII (10–100 µg/ml), and influenza A/X31 virus antigen (a kind gift of Dr. Skehel, NIHS-Mill Hill, U. K., 0.5 µg/ml). Some cultures were also stimulated by pokeweed mitogen (PWM, 25 µl/ml—Gibco Laboratories).

At day 4, the culture medium was removed and replaced by 0.2 ml of fresh medium (RPMI 1640 + fetal calf serum + antibiotics, amphotericin B, and glutamine), without further addition of antigen or mitogen. Cultures were stopped at day 7 except when otherwise stated.

Proliferative responses were evaluated by incorporation of tritiated thymidine for the last 18 h of culture and by enumeration of live harvested cells using the trypan blue exclusion method.

Detection of antimannan ACC. Smears of harvested cells after cytocentrifugation were fixed in ethanol, washed with phosphate-buffered saline, then covered with labeled antigen (1:100 dilution of stock solution) for 30 min, washed in phosphate-buffered saline, and then fixed again in ethanol. Intracytoplasmic binding of labeled antigen ([³H]mannan or

[^3H]SIII) was revealed by radioautography after an 8-d exposure in emulsion (K5 Ilford, Essex, England). Smears were then stained with methyl green pyronin. The percentage of labeled cells (ACC) was counted at least on 1,000 cells. All smears were observed in blind.

Detection of immunoglobulin-containing cells (ICC) was performed with rhodamine- or fluorescein-labeled monospecific anti- μ , - γ , or - α heavy chain antisera (Nordic Laboratories) either on smears prepared for radioautography just before exposure in emulsion or on separate smears. Double analysis of the smears allowed to visualize in [^3H]mannan-binding cells intracytoplasmic Ig although immunofluorescence was dull when using that procedure as compared with control smears. Positive cells were called ICC-ACC.

Detection of antibodies in supernatants. Specific antimannan antibody content of culture supernatants was determined by ELISA (22). The supernatants were harvested either from day 4 to 7, from day 6 to 7, or from day 7 to 10. Antigens, mannan (at a concentration of 250 $\mu\text{g}/\text{ml}$), or A/X31 influenza virus antigen (at a concentration of 50 $\mu\text{g}/\text{ml}$) was adsorbed on flat-bottom microtiter plates (Linbro Chemical Co., Hamden, CT) for 1 h at 37°C. In some experiments, MBSA was used to coat microtiter plates at a dose of 250 $\mu\text{g}/\text{ml}$. Plates were rinsed twice with HBSS, then once with HBSS containing 1% BSA (Sigma Chemical Co.). Non-specific binding was blocked by a further incubation with HBSS-BSA. Plates were washed again as mentioned above, then culture supernatants (100 μl) were added to each well for 1 h at 37°C. After three further washings, 100 μl of alkaline phosphatase-coupled goat anti-human IgM (Miles Laboratories, Yeda, Israel, final concentration 1:500) or IgG (Miles Laboratories—final concentration 1:1,600) was added to each well for 1 h at 37°C. Plates were then washed again twice with HBSS, once with HBSS-BSA and three times with magnesium-containing carbonate buffer pH 9.6. 100 μl of nitrophenyl phosphate substrate for alkaline phosphatase

(Sigma Chemical Co.) at a final concentration of 1 mg/ml in carbonate buffer was added to each well. Plates were incubated for 1 h at 37°C. The absorbance at 405 nm of each well was read on a Titertek multiscan (Flow Laboratories, McLean, VA). Results from quadruplicate wells expressed in optical density were compared with a reference serum obtained from a *Candida albicans*-sensitized subject. The standard curve for the ELISA using antimannan-immunoglobulins M or G is shown in Fig. 1. Antibody concentration is expressed as \log_2 dilutions of the standard reference. A straight line was obtained when logit transformed OD_{405} was plotted against \log_2 of dilution. 1 unit was arbitrarily defined as the amount of antimannan-specific IgM or IgG present in a 1:10,000 dilution of the reference serum since it gave a significant level of OD_{405} . The standard deviation of quadruplicate determinations of the reference serum was <5%. Furthermore, an excess of free mannan completely blocked the response in the assay, indicating the specificity of the ELISA.

RESULTS

Two methods were used for the detection of antimannan antibody production: enumeration of antimannan ACC and titration of antimannan antibodies in supernatants by ELISA. Results of both methods will be successively described.

Generation of antimannan ACC by human lymphocytes

Antigen requirement. PBL obtained from most normal *Candida albicans*-sensitized volunteers, (i.e.,

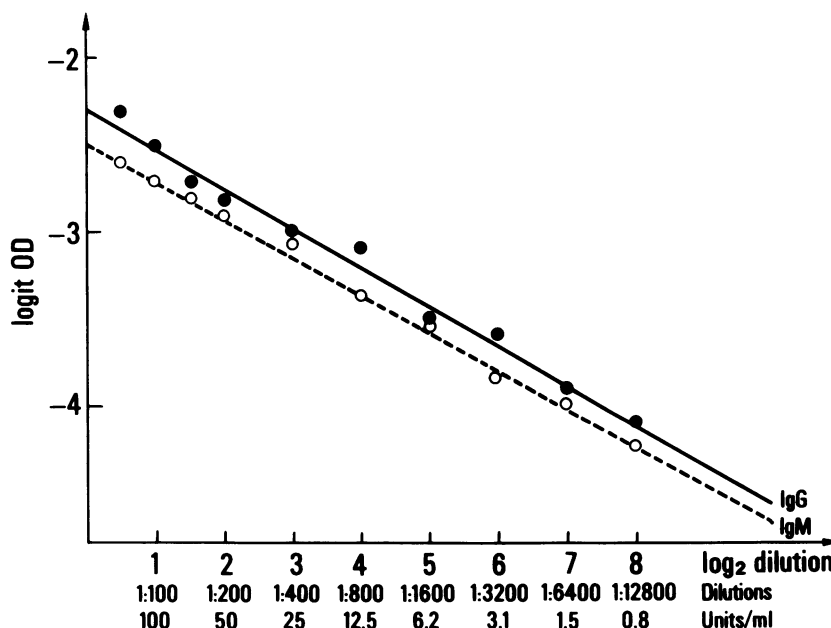


FIGURE 1 Standard curve of optical density vs. twofold dilutions of the reference serum. The curve was linearised by logit transformations of each optical density. Arbitrarily, we defined the dilution 1:100 as 2 ($\log_2 2 = 1$), 1:200 as 4, etc. . . . IgM antimannan, —●— IgG antimannan ○—○.

35/40 subjects who exhibited serum antibodies), proliferated and matured into antimannan ACC, in cultures initiated with mannan absorbed on MBSA (mannan-MBSA). Mannan alone whatever the dose used (5–250 $\mu\text{g/ml}$) and the duration of the culture (3–10 d) was unable to induce proliferation and antimannan ACC generation. Mannan-MBSA was able to elicit the generation of ICC of the IgM or the IgG class but not of the IgA class. The number of ACC correlated with the number of total recovered cells and the incorporation of [^3H]thymidine (Table I). The threshold concentration of mannan-MBSA inducing proliferation and maturation was 10 $\mu\text{g/ml}$, the optimal concentration was 50 $\mu\text{g/ml}$ for both, while a proliferation only was observed when cultures were stimulated with 150 or 200 $\mu\text{g/ml}$ mannan-MBSA. At higher doses of antigen (up to 500 $\mu\text{g/ml}$) no response was observed. Subsequently, cell cultures were stimulated with 50 $\mu\text{g/ml}$ mannan-MBSA.

However, in 5 out of 40 sensitized subjects tested, no generation of ICC or ACC was found despite the presence of a cell proliferation whatever the dose of mannan-MBSA used (5–250 $\mu\text{g/ml}$) and the duration of the culture (5–10 d).

MBSA alone, at dose ranging from 1 to 100 $\mu\text{g/ml}$, constantly failed to induce cell proliferation and B cell

maturation into ICC and ACC from mannan-MBSA responder PBL (Table I). By an indirect immunofluorescence test (using MBSA, sheep anti-MBSA antiserum revealed by a rhodamine-labeled goat anti-sheep Ig antiserum), no anti-MBSA ACC was observed neither in MBSA nor in mannan-MBSA-stimulated cultures. Finally, PWM, although it induces a strong proliferation and generation of ICC, was shown to poorly trigger sensitized lymphocytes into antimannan ACC (Table I).

Nature of the ACC. Antimannan-containing cells were easily detectable on radioautography smears after staining with methyl green pyronin as large pyroninophilic cells (size 15–30 μm) (Fig. 2). The number of silver grains observed varied from cell-to-cell but the majority of cells were strongly labeled. Analysis of labeled cells showed that they were OKT3(–), OKM1(–), and unable to phagocytize latex beads. Moreover, they bore surface immunoglobulins (IgM or IgG) and contained intracytoplasmic immunoglobulins (IgM or IgG), but never both classes in the same cell as demonstrated by double immunofluorescence. In mannan-MBSA (10–100 $\mu\text{g/ml}$)-stimulated cultures performed in flat-bottom microwells, there was only a slightly higher number of ICC than of ACC (Table I) and intracytoplasmic immunofluorescence technique per-

TABLE I
Generation of Antimannan ACC by Human Lymphocytes Stimulated by Mannan-MBSA

Cultures stimulated with	Number of experiments performed	Proliferative response			Number of cells containing	
		cpm ($\times 10^{-3}$)	Number of recovered cells ($\times 10^{-3}$)	Number of antimannan ACC ($\times 10^{-3}$)	IgM ($\times 10^{-3}$)	IgG ($\times 10^{-3}$)
0	35	3 \pm 2	150 \pm 86	<0.1	0.4 \pm 0.5	0.3 \pm 0.4
Mannan-MBSA (10 $\mu\text{g/ml}$)	7	60 \pm 16	545 \pm 116	7.8 \pm 2.3	2.4 \pm 0.8	3.8 \pm 1.5
Mannan-MBSA (50 $\mu\text{g/ml}$)	35	90 \pm 21	707 \pm 135	16.3 \pm 4.2	11.7 \pm 5.7	8.5 \pm 6.6
Mannan-MBSA (100 $\mu\text{g/ml}$)	7	48 \pm 15	682 \pm 123	8.9 \pm 2.8	2.4 \pm 1.2	4.4 \pm 0.9
Mannan-MBSA (500 $\mu\text{g/ml}$)	3	4 \pm 3	110 \pm 90	<0.1	0.5 \pm 0.3	0.1 \pm 0.2
Mannan (40 $\mu\text{g/ml}$)	6	7 \pm 3	277 \pm 80	<0.1	0.3 \pm 0.1	0.4 \pm 0.2
MBSA (10 $\mu\text{g/ml}$)	6	5 \pm 2	170 \pm 103	<0.1	0.2 \pm 0.1	0.3 \pm 0.2
PWM (25 $\mu\text{l/ml}$)	20	185 \pm 66	985 \pm 236	<0.5	218.2 \pm 46.3	214.7 \pm 39.9

Cultures were stimulated either with mannan absorbed on MBSA at a ratio 4:1, mannan alone, MBSA alone or PWM, for 7 d in flat-bottom microwells. Proliferative response was evaluated by [^3H]thymidine incorporation (cpm $\times 10^{-3}$) and by the number of recovered cells ($\times 10^{-3}$) for 1×10^6 input cells. The number of antimannan ACC was evaluated by enumerating the cells fixing [^3H]mannan and revealed by radioautography ($\times 10^{-3}$ for 10^6 input cells). Generation of ICC was measured by counting the number of IgM- or IgG-containing cells detected by intracytoplasmic immunofluorescence ($\times 10^{-3}$ for 10^6 input cells). Results are expressed as the means \pm 1 SD of the responses obtained with PBL from different *Candida albicans*-sensitized responder donors. The differences between unstimulated cultures and mannan-MBSA (10, 50, and 100 $\mu\text{g/ml}$) were significant ($P < 0.001$ for proliferation (thymidine incorporation and number of recovered cells) and for ACC generation). The differences between unstimulated cultures and mannan-MBSA at high doses (500 $\mu\text{g/ml}$) were not significant ($P > 0.3$ for proliferation (thymidine incorporation and number of recovered cells) and for ACC generation).

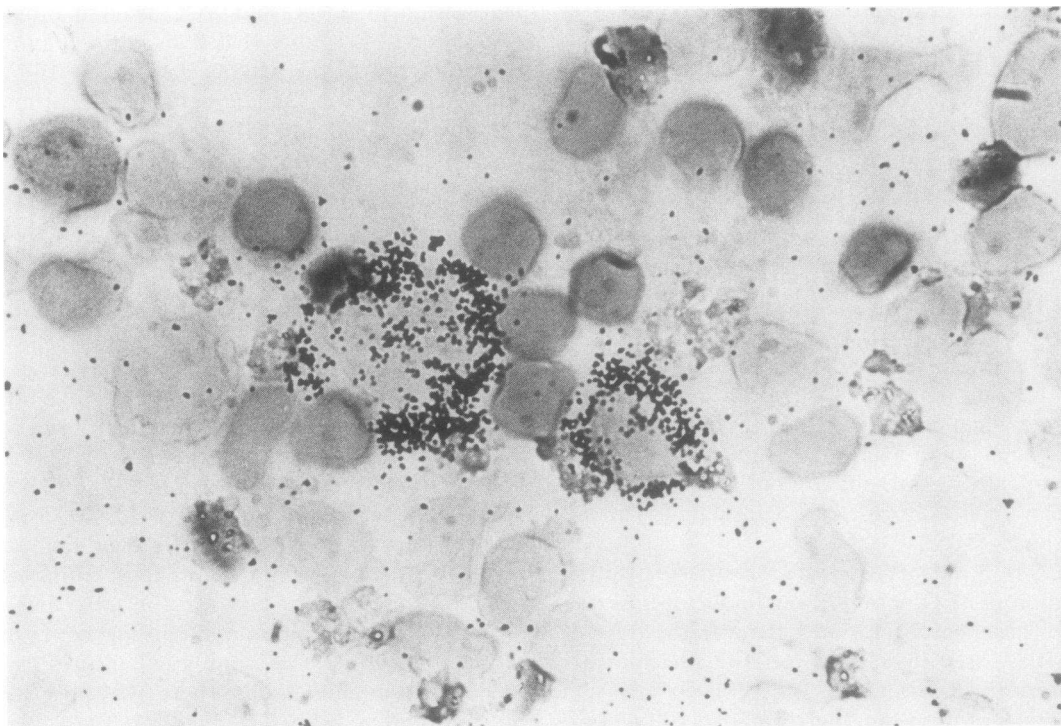


FIGURE 2 Antimannan ACC were detected at the end of 7-d mannan-MBSA (50 $\mu\text{g}/\text{ml}$)-stimulated cultures by radioautography using [^3H]mannan after an 8-d exposure in emulsion. Smears were coloured with methyl green pyronin.

formed together with radioautography on the same smears indicated that only a few ICC did not bind [^3H]mannan. On the contrary, in cultures performed in round-bottom microwells, the number of ICC doubled while the number of ACC did not change. Subsequently, all cultures were performed in flat-bottom microwells.

Kinetics of the response. The kinetics of the antimannan ACC generation was studied in five sensitized responder subjects, and results are shown in Fig. 3. A proliferative response judged by [^3H]thymidine incorporation or by enumeration of live harvested cells appeared at day 3, peaked at days 5–6, and was null at day 9. Antimannan ACC appeared at day 6 and we showed, by coupling intracytoplasmic immunofluorescence and radioautography, that a large majority of ACC-ICC contained IgM. At day 7 and 8, generation of ACC-ICC of both IgM and IgG class was maximal and dramatically decreased thereafter.

Reproducibility of the response. Table II gave results obtained in three subjects studied repeatedly within 1 yr and showed that cells from responder subjects did constantly mature into ACC. The proportion of IgM and IgG-ICC was variable from subject to subject but was reproducible in a given subject. For the

subjects 1 and 2, the number of IgM-ICC was repeatedly superior to the number of IgG-ICC. On the contrary, PBL of subject 3 differentiated preferentially and repeatedly into IgG-ICC. Some experiments, where immunofluorescence was coupled with radioautography, demonstrated that the proportions of IgM-ACC and IgG-ACC were rather constant in a given subject.

Specificity of the ACC. PBL from most of *Candida albicans*-sensitized subjects were differentiating in vitro into ACC in presence of mannan-MBSA. However, PBL from unsensitized subjects, i.e., blood donors without detectable serum anti-*Candida* antibodies, or isolated from cord blood, never proliferated nor matured into ACC and ICC, whatever the dose of mannan-MBSA used (5–200 $\mu\text{g}/\text{ml}$) and the duration of cultures (5–10 d), indicating that mannan-MBSA did not induce a nonspecific polyclonal Ig production.

The specificity of the intracytoplasmic labeling by [^3H]mannan of mannan-MBSA-stimulated cells was studied in different ways. Firstly, cell smears were incubated with a large excess (30–100-fold of cold mannan before the addition of [^3H]mannan. In this condition, ACC were no more detectable by radioautography.

On the contrary, addition of a large excess of an

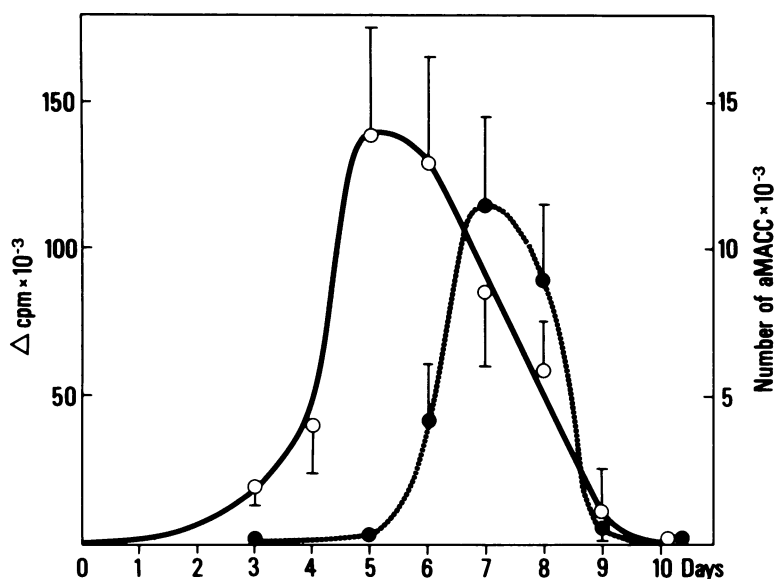


FIGURE 3 Time-curve response of sensitized PBL to mannan-MBSA (50 $\mu\text{g/ml}$). Proliferation (○) was evaluated by [^3H]thymidine incorporation ($\Delta \text{cpm} \times 10^{-3}$). Generation of antimannan ACC (●) was expressed by the number of [^3H]mannan-binding cells for 10^6 cultured PBL. Results are the means of five experiments ± 1 SD.

unrelated cold polysaccharide SIII before incubation with [^3H]mannan did not inhibit intracytoplasmic binding of [^3H]mannan.

TABLE II
Reproducibility of the Generation of Antimannan ACC

Subjects	Proliferative response		Number of antimannan ACC ($\times 10^{-3}$)	Number of cells containing	
	cpm ($\times 10^{-3}$)	Number of recovered cells ($\times 10^{-3}$)		IgM ($\times 10^{-3}$)	IgG ($\times 10^{-3}$)
1	190	1,225	16.0	12.0	10.6
	80	635	12.7	9.7	4.3
	35	620	5.8	5.1	2.5
	125	1,200	13.6	14.0	8.1
2	20	535	5.3	4.0	1.4
	30	750	13.1	13.5	1.1
3	20	660	7.6	3.7	4.7
	15	690	13.5	4.4	10.7
	75	835	8.2	2.9	4.2
	40	565	3.9	1.4	2.3

Three different *Candida albicans*-sensitized responder donors were repeatedly studied at different times, with an interval of several weeks (subject 1) or months (subjects 2 and 3). Cultures were stimulated with an optimal dose of mannan-MBSA (50 $\mu\text{g/ml}$) in flat-bottom microwells for 7 d. The proportions of IgM- and IgG-ICC in a given subject were rather constant. Similar results were observed by enumerating IgM and IgG-ACC by coupling immunofluorescence and radioautography on the same smears. Similar results were obtained in duplicate smears. Results are expressed for 10^6 initially cultured cells.

Secondly, PBL from recently immunised subjects (15–30 d after immunisation) and stimulated by polysaccharide SIII proliferated and generated specific anti-SIII-ACC of both IgM and IgG classes, as revealed by the use of [^3H]SIII in radioautography. SIII-induced blast cells that were able to bind [^3H]SIII were not labeled by [^3H]mannan (Table III). Conversely, mannan-MBSA-stimulated blast cells that were able to bind [^3H]mannan, were not labeled by [^3H]SIII.

TABLE III
Generation of Antimannan or Anti-S III ACC in One Sensitized Subject

Cultures initiated with	Proliferative response	Number of cells containing antibodies to	
		Mannan ($\times 10^{-3}$)	S III ($\times 10^{-3}$)
<i>cpm $\times 10^{-3}$</i>			
MBSA-mannan	750	15.9	<0.1
S III	412	<0.1	4.5
A/X31 virus	580	<0.1	ND

PBL from a subject sensitized with *Candida albicans*, influenza virus A, and recently immunized with pneumococcal polysaccharide (SIII) (15 d before) were cultured for 7 d with mannan-MBSA (50 $\mu\text{g/ml}$), SIII (100 $\mu\text{g/ml}$), or A/X31 (0.5 $\mu\text{g/ml}$) in flat-bottom microwells.

Results of a representative experiment among four are expressed for 10^6 initially cultured cells.

Furthermore, as mentioned above, only a few PWM-induced ICC bound [^3H]mannan and influenza-virus A/X31-induced ICC were not labeled by [^3H]mannan. Altogether, these data indicate that mannan-MBSA-induced blast cells are specifically labeled by [^3H]mannan.

Detection of antimannan antibodies in culture supernatants

In cultures of PBL from sensitized donors, stimulated with mannan absorbed on MBSA immediately before being added to cultures, IgM and IgG antimannan antibodies were easily detected by a solid phase enzyme-linked immunoassay. Table IV shows results observed with PBL from different sensitized subjects. In unstimulated or mannan-stimulated (5–250 $\mu\text{g}/\text{ml}$) cultures, antibody titer was very low as well for IgM as for IgG. With mannan-MBSA (for concentrations ranging from 10 to 100 $\mu\text{g}/\text{ml}$) a significantly higher titer ($\times 8$) of both IgM antimannan antibodies or IgG antimannan antibodies were found. The reproducibility of the ELISA was good since the variation between quadruplicate wells of a given cul-

TABLE IV
Production of Antimannan Antibodies in Cultures Stimulated With Mannan-MBSA

Cultures stimulated with	Number of experiments performed	Specific antibody concentration	
		IgM	IgG
		U/ml	
0	15	1.7 \pm 1.4	2.3 \pm 2.9
MBSA-mannan (50 $\mu\text{g}/\text{ml}$)	15	17.1 \pm 10.3	18.8 \pm 17.8
Mannan (40 $\mu\text{g}/\text{ml}$)	5	1.8 \pm 2.4	1.4 \pm 1.6
MBSA (10 $\mu\text{g}/\text{ml}$)	5	2.2 \pm 1.9	0.8 \pm 0.8
PWM (25 $\mu\text{g}/\text{ml}$)	5	2.8 \pm 1.8	3.2 \pm 3.1

Cultures were stimulated with either mannan absorbed with MBSA at ratio 4:1, mannan alone, MBSA alone, or PWM for 7 d in flat-bottom microwells. Supernatants from day 4 to 7 were harvested and titration of specific antimannan antibodies was performed by ELISA.

Results are the means \pm 1 SD obtained in several different *Candida albicans*-sensitized responder subjects.

Results are expressed in units per milliliter by comparison with the 1:10,000 dilution of a reference serum from a *Candida albicans*-sensitized donor, according to the linear relationship between the logit of the optical density and the log₂ of the serum dilution (Fig. 1).

The differences between unstimulated cultures and mannan-MBSA (50 $\mu\text{g}/\text{ml}$)-stimulated cultures were significant, as well for IgM antibody production ($P < 0.001$) as for IgG antibody production ($P < 0.001$). In supernatants of PBL from one subject repeated every 2 wk, variations of specific antibody concentrations were twofold at most.

ture was $<20\%$ and the variation between two different determinations of the same well was $<10\%$. Furthermore, when cultures of PBL from the same sensitized donor were repeated at several days intervals, results were comparable (twofold variation at most).

When mannan was omitted in the first step of the ELISA, no binding of IgM or IgG did occur (data not shown). It appears that 50 $\mu\text{g}/\text{ml}$ of mannan-MBSA was the optimal concentration for the specific antibody production as for the generation of ACC. No difference was observed between supernatants collected from either day 4 to 7, 6 to 7, or 7 to 10. All subsequent antibody titrations have thus been performed in supernatants of cultures stimulated by 50 $\mu\text{g}/\text{ml}$ of mannan-MBSA and collected from day 4 to 7. When cultures were stimulated with MBSA alone (1–100 $\mu\text{g}/\text{ml}$), no antimannan antibody could be detected in supernatants. Moreover, when MBSA itself, instead of mannan, was coated on the plastic wells used for ELISA, no anti-MBSA antibody could be detected in supernatants of cultures induced either by MBSA or mannan-MBSA, confirming the results, obtained by intracytoplasmic immunofluorescence using an anti-serum to MBSA. A few cultures were run in human serum from nonsensitized donors in order to detect anti-MBSA antibodies. In these conditions, no anti-MBSA antibodies were found although antimannan ACC were normally generated. In mannan-MBSA-stimulated cultures of PBL isolated from unsensitized donors or from cord blood, no antimannan antibody could be detected, whatever the antigen concentration used (5–100 $\mu\text{g}/\text{ml}$) or the culture duration (7–10 d). These data confirmed again the lack of nonspecific polyclonal activation induced by mannan-MBSA.

When PWM was used to drive cultures in sensitized subjects, no detectable antimannan antibodies were found in 7- or 10-d supernatants.

The production of antimannan antibody was shown to be specific. Indeed, in cultures of PBL isolated from A/X31 influenza virus-sensitized donors stimulated with A/X31 virus, no antimannan antibodies were detected in supernatants, while large amounts of IgG anti-A/X31 were found (Table V). Conversely, when PBL from the same donors were cultured with mannan-MBSA, no antibodies to A/X31 were detected, while both IgM and IgG antimannan antibodies were produced.

As indicated in Table VI, the generation of ACC enumerated by radioautography closely correlated with the production of antibodies determined by the ELISA method. For example, in 7-d cultures of PBL from subjects 1 to 3, a large number of IgM and IgG ACC were detected as well as high titers of IgM- and IgG-specific antibodies were found in culture supernatants. A preponderant generation of IgM ACC in

TABLE V
Specificity of the Antimannan Antibody Production

Cultures stimulated with	Specific antibody concentration			
	Mannan		A/X31	
	IgM	IgG	IgM	IgG
	U/ml			
MBSA-mannan	15	24	<1	1
A/X31 influenza virus	0	1	1	60

Cultures were initiated with mannan-MBSA (50 µg/ml) or with A/X31 influenza virus (0.5 µg/ml) for 7 d in flat-bottom microwells. Supernatants from day 4 to 7 were collected for titration of specific antibodies by ELISA.

Results, obtained from cultures of PBL from one subject sensitized donor to *Candida albicans* and to influenza virus A, are expressed in units per milliliter, as compared with the 1:10,000 dilution of the reference seum, isolated from a *Candida albicans*-sensitized subject and from a A/X31 influenza virus-sensitized donor. The standard deviation of the means of quadruplicates was always <20%. One representative experiment among three is shown.

cultures of PBL from adults 4–6 was associated with detection of antibodies exclusively of the IgM class in supernatants. Finally, in cultures of PBL from adult 7, only a few (but significant) number of ACC was generated whereas no detectable antibody was detected in supernatants.

T cell dependency of antimannan antibody production

T cell dependency of specific antimannan antibody production was studied by performing E-rosette fractionation of PBL. As shown in Table VII, E(+) preparations were neither able to differentiate into antimannan ACC nor to produce antibodies when cultured for 7 d in presence of 50 µg/ml of mannan-MBSA. Likewise, E(–) cell populations, prepared by a depletion of T lymphocytes using OKT3 in presence of complement, did not produce any antimannan-ACC nor specific antimannan antibody in supernatants. These negative results were still observed after depletion of E(–) preparations of the excess of monocytes by three successive steps of adherence to plastic. However, addition of autologous E(+) lymphocytes to E(–) cells allowed the generation of antimannan ACC and of supernatant antibodies. This helper effect appeared at a low E(+):E(–) ratio of 1:4 but was maximal at a ratio of 1:1, and decreased at higher ratios. Irradiation with 1,000 rad of the E(+) lymphocytes before being added to autologous E(–) cells completely abolished the production of antimannan ACC and antimannan antibodies in supernatants even when the ratio between

irradiated E(+) and E(–) cells was as high as 6:1. The same irradiated E(+) cells were able to help B cells (ratio E(+):E(–) = 4:1) to produce immunoglobulins when stimulated by PWM (data not shown). In cultures mixing E(+) and E(–) cells from two unrelated HLA-different donors (ratio 1:1) no helper activity was observed. However, addition of AC autologous to E(+) lymphocytes restored a normal generation of specific antimannan ACC. This effect was constantly observed, whatever the ratio AC:E(+) (from 0.15:1 to 0.3:1) and was resistant to irradiation (3,000 rad) of the AC. The control culture, mixing E(+) and autologous AC was negative, indicating the absence of a sufficient contamination by B lymphocytes of AC. In co-cultures mixing E(+) lymphocytes from one donor, and E(–) plus AC isolated from a same second donor, no production of ACC could be detected (data not shown). Finally, a production of ACC could be detected in mannan-MBSA-stimulated cultures mixing E(+) and E(–) cells from related HLA semi-identical donors (mother and daughter) (Table VII).

TABLE VI
Correlation between Antimannan Antibody-producing Cells and Antimannan Antibody Concentrations in Culture Supernatants

Subject	Number of cells containing intracytoplasmic			Antibody concentration in supernatants	
	Antibodies to mannan ($\times 10^{-3}$)	Immunoglobulins		IgM	IgG
		IgM ($\times 10^{-3}$)	IgG ($\times 10^{-3}$)		
U/ml					
1	11.4	6.5	5.2	23	25
2	22.9	5.1	21.0	16	95
3	18.1	3.0	17.5	15	70
4	5.7	3.1	1.4	15	5
5	10.0	8.4	1.1	40	4
6	3.6	2.5	0.8	14	5
7	2.8	1.5	1.7	4	3

Cultures were stimulated with mannan-MBSA (50 µg/ml) for 7 d in flat-bottom microwells.

Results of antimannan ACC and of ICC of the IgM or IgG class are expressed for 10^6 initially input cells. Supernatant antibody concentrations are expressed in units per milliliter as compared with the reference serum. The standard deviation of the means of quadruplicates was always <20%.

Results from seven representative experiments are shown.

In unstimulated cultures of each subject, production of immunoglobulin or antimannan antibodies was negligible (number of ICC $< 1 \times 10^{-3}$, number of ACC $< 0.1 \times 10^{-3}$ and supernatant antibody concentrations < 3 U/ml).

The correlation coefficient between the detection of cells containing intracytoplasmic antibodies and the detection of supernatant antibodies was $r = 0.92$, $P < 0.01$.

TABLE VII
Cellular Requirements for T Cell Dependency of Antimannan Antibody Production in Mannan-MBSA-stimulated Cultures

Cultured cells	Number of experiments performed	Antibody containing cells ($\times 10^{-5}$)	Supernatant antibody concentrations	
			IgM	IgG
			U/ml	
Unseparated PBL	10	12.3 \pm 5.4	16.9 \pm 10.1	23.7 \pm 12.1
E(+)	10	<0.1	1.2 \pm 1.7	1.9 \pm 1.5
E(-) [*]	10	<0.1	2.3 \pm 0.9	1.4 \pm 2.1
E(+) + autologous E(-) [*] †	10	14.0 \pm 8.9	18.5 \pm 6.1	20.5 \pm 11.8
Irradiated E(+) [§] + autologous E(-) [*] †	10	<0.1	3.4 \pm 2.3	2.5 \pm 0.8
E(+) + allogeneic E(-) [*] †	10	<0.1	1.8 \pm 1.5	0.8 \pm 0.5
E(+) + autologous AC				
+ allogeneic E(-) [*] †	6	6.8 \pm 4.1	ND	ND
E(+) + autologous AC	6	<0.1	ND	ND
E(+) + semi-identical E(-) [*] †	4	11.2 \pm 3.5	ND	ND

Experiments were performed with total or separated PBL from normal volunteers and E(-) cells isolated from either unrelated HLA-different or related HLA-semi-identical (mother or daughter) controls.

Cultures were stimulated with mannan-MBSA (50 μ g/ml) for 7 d in flat-bottom microwells. Results of antibody containing cells are given for 10^6 initially cultured cells and results of supernatant antibodies are given in units per milliliter as compared with the reference serum. The standard deviation of the means of quadruplicates was always <20%.

^{*} E(-) preparations were treated with OKT3 and complement. Similar results were obtained after partial depletion of monocytes by three successive steps of adherence on plastic.

† E(+) lymphocytes were added to E(-) cells at ratio 1:1. A suboptimal helper effect of E(+) lymphocytes was observed at lower ratio (1:4) or at higher ratio (4:1).

§ E(+) lymphocytes were irradiated at 1,000 rad. Similar results were obtained with E(+) lymphocytes irradiated at 2,000 or 3,000 rad, or with higher ratios of E(+)/E(-) (until 6:1).

|| Around 15×10^3 AC were added to 100×10^3 E(+) lymphocytes. Similar results were obtained with 30×10^3 AC added, or with irradiated (3,000 rad) AC. E(+) and E(-) cells were added to flat-bottom microwells in which AC have been previously isolated.

DISCUSSION

We have developed in the present study an in vitro assay that detects human B cells-producing antibodies to mannan, a polysaccharide from *Candida albicans*. This system provides a new way of testing specific in vitro antibody production in humans. Cultures of PBL obtained from sensitized donors were stimulated with purified mannan absorbed with MBSA. Two methods were used in parallel to evaluate the antimannan antibody production. Antimannan ACC were detected by a radioautographic technique using tritiated mannan labeled by catalytic exchange. This method provides a tritiated mannan with high specific activity, stable, and with an unmodified structure (23). In cultures performed in flat-bottom microwells, the proportion of antimannan ACC among ICC was remarkably high as compared with that observed in previously

published in vitro antibody production assays (3, 4, 6) but was comparable to the data observed in the keyhole limpet hemocyanin system at low dose of antigen (11). However, mannan is known to be a strong immunogen (21) and the radioautographic method used to detect the ACC can be more sensitive than hemolytic plaque-forming cell assay and than the immunofluorescence technique used to detect the ICC. The radioautographic technique takes a longer time for detecting in vitro production of antibodies than other techniques, such as hemolytic plaque-forming cells (3, 4), but it gives a direct identification of the antibody-producing cells.

The detection of large amounts of antimannan antibodies by an ELISA method confirmed that mannan truly triggered B cells to produce specific antibodies. A relatively high concentration of mannan was necessary for the first step of the ELISA, indicating a low-

binding affinity of mannan for plastic. However, the mannan binding was stable enough to allow the titration of specific antibodies.

Two major issues about our assay need more discussion in order to delineate its significance. That is the nature of the antigen and the specificity of the antibody response. The antigen preparation we used contained above all mannan. However, there is a tiny contamination with protein. One should thus envisage a possible antiproteomannan response in this system, especially as relatively high concentrations of antigen were required for the induction of antibody production. However, this hypothesis appears unlikely since the protein contaminant was <4% of the antigen preparation. Furthermore, we showed that the antigen preparation alone did not trigger proliferation and maturation into ACC. On the contrary, other *Candida albicans* antigen preparations like Candida metabolic antigen that contains higher amounts of protein (>10%) induced a strong proliferation of sensitized PBL (14). It was obvious that the antigen preparation used in this study need to be extemporaneously absorbed to MBSA to trigger cell proliferation and antibody production. It seems likely that MBSA and mannan, a neutral polysaccharide, form aggregates (despite the lack of electrolytic linkage binding), which may contribute to the antigen presentation to monocytes.

The generation of antimannan antibodies appears to be a specific immune response and not a mitogen-induced polyclonal immunoglobulin production like that observed with PPD (25) or different bacterial cell walls containing peptidoglycans (26), since nonresponder B cells were not induced to differentiate into ICC. Evidence of the specificity of the response was given in experiments in which it was possible to obtain anti-S III ACC by using cells from donors recently immunized with the S III pneumococcal polysaccharide. A pulse of unlabeled mannan, but not S III, nearly abrogated the binding of labeled mannan to ACC. As measured by ELISA, mannan-MBSA did not induce antiinfluenza A antibody production by B cells from influenza A primed donors and, conversely, influenza A virus did not induce a detectable antimannan antibody production. Thus, in both methods used to study antimannan antibody production, there is a strong evidence for the specificity of the response. However, like in the keyhole limpet hemocyanin system (11), the use of round-bottom microwells induce a nonspecific polyclonal component in the response. On the other hand, a polyclonal stimulation of B cells by PWM resulted in a very low percentage of antimannan ACC among ICC, showing that ICC were not nonspecifically stained by [³H]mannan. It was surprising to observe that PWM did not induce a higher number of antimannan ACC nor antimannan antibodies in cul-

ture supernatants as previously observed for other antigenic systems (9, 11). Since PWM acts only on a given B cell subset (27, 28), one can envisage that the mannan-responding B cells mostly belong to another population. It would be interesting to know if B cells specific for other polysaccharides behave identically.

In vitro antimannan ACC generation was only observed in sensitized donors whose lymphocytes were able to proliferate in presence of *Candida* antigens. These data clearly show that antimannan ACC generation is a secondary response like the systems described for tetanus toxoid antigen (9), influenza virus (7, 8), or keyhole limpet hemocyanin (10, 11). Donor lymphocytes with positive proliferations but with an absence of antibody production to mannan were rarely observed. No common feature could be observed in those donors to explain these negative results. They could be due to an absence of recirculating specific memory B cells in the blood (8). The system presently described is however reliable for studying antibody production since 35 out of 40 donors tested had B lymphocytes able to respond to mannan. Our observation of an in vitro strong antibody production to mannan parallels the in vivo findings of the predominance of antimannan antibodies in the serum of patients with candidiasis (24). One should notice that there was a large proportion of B cells producing antimannan antibodies of the IgM class among antibodies, as shown by the detection of IgM and IgG antimannan ACC and antibodies in MBSA-mannan-stimulated cultures. This could appear as an uncommon feature for a secondary antibody response (29) but has been already described in the keyhole limpet hemocyanin system (11). Furthermore, the polysaccharide antigen could preferentially trigger the production of IgM antibodies (30). The kinetics of the generation of antimannan ACC was however comparable to that already described for other secondary responses (7, 11).

One interesting aspect was the possibility to raise the question of the T cell dependency of antimannan antibody responses in humans. Indeed, in the mouse, antibody production to most polysaccharides is thymus independent (31). In the hapten-carrier system trinitrophenyl of Ficoll in mice, it was also shown that antibody production was T independent, although B cell responses could be augmented and qualitatively changed by T cells (32). Our results clearly show that the generation of antimannan ACC required the presence of T cells even for IgM antibody production. However, one cannot distinguish between the existence of helper T cells for mannan itself or an equivalent of a hapten-carrier help in which T cells would recognize MBSA and help B cells to produce antimannan antibodies.

On the other hand, our experiments in which E(+)

and E(-) cells from different donors were mixed suggested the existence of an allogeneic restriction. A similar observation has been already shown in the *in vitro* antibody assay for tetanus toxoid (33) and influenza virus (34). In the influenza virus system, cells interacted in a negative manner since irradiation of T cells, which abolished allogeneic suppression, permits the expression of a specific help across an HLA barrier. The radiosensitivity of specific help in our system did not allow to study an allogeneic activation of suppressor T cells. We obtained however results that strongly suggest an actual genetic restriction located at T-macrophage interaction, as already suggested in an other antigenic system (35). In fact, addition of autologous adherent cells to T lymphocytes permits a normal help to allogeneic B cells.

We showed that T cells involved in such an interaction are radiosensitive. This result may be related to the observation of a radiosensitive helper T cell subset described in the PWM system (36, 37). However, in that polyclonal response, radiosensitive helper T cells seems to act only at low T/B cell ratios. In our system, the specific help was totally radiosensitive, even at high T/B ratios. This result is comparable with that observed in the keyhole limpet hemocyanin or tetanus toxoid system where the marked reduction of specific helper T cell function after irradiation has been shown not to be ablated at high T/B cell ratios (38).

The development of an *in vitro* system for specific antibody production by human PBL to a polysaccharide antigen opens the way for studies that have not yet been possible, about the fine regulation of antibody production to polysaccharides in humans, as most adults have already been primed to mannan from *Candida albicans*. This system should provide also interesting information in several pathological conditions.

ACKNOWLEDGMENTS

We are grateful to C. de Bièvre and Prof. F. Drouhet (Institut Pasteur, Paris, France) for the kind gift of mannan, to Dr. Jaton (Genève, Switzerland) for providing pneumococcal S III, and to B. Fournet (Institut Biochimie, Lille, France) for biochemical studies of antigen preparations. We thank Mrs. C. Barbat, M. Forveille, and A. M. Laharie for excellent technical assistance, and B. Delpit for typing the manuscript.

This work was supported by Institut National de la Santé et de la Recherche Médicale and by Conseil Scientifique Université Paris V.

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