HUMAN IMMUNE RESPONSE TO GROUP A STREPTOCOCCAL CARBOHYDRATE (A-CHO)

I. Quantitative and Qualitative Analysis of the A-CHO-specific B Cell Population Responding in Vitro to Polyclonal and Specific Activation

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The induction, in vitro, of human antibody responses to non-MHC[1] (major histocompatibility complex) antigens has been described for only a few soluble protein antigens, including tetanus toxoid (1), influenza virus proteins (2), and keyhole limpet hemocyanin (3). In contrast, the in vitro induction of human antibodies specific to carbohydrate antigens has not been systematically analyzed. Responses to carbohydrate determinants are involved in the defense against bacterial infections, parasites, and perhaps in the recognition of tumor-associated antigens (4). As a model for studying the human anticarbohydrate response and its regulation in vitro, we chose the response to the group-specific carbohydrate (A-CHO) of A streptococci (Strep A), for the following reasons: (*a*) Remarkable amounts of anti–A-CHO antibodies were found in sera of most human adults (5, 6, 7) suggesting a ubiquitous anamnestic response in the human population. Therefore, in vitro studies do not require artificial immunizations. (*b*) The anti–A-CHO response in rabbits and in mice has been extensively analyzed both in vivo and in vitro (8–10). Beside the knowledge on specificity and heterogeneity of T and B cells involved in this response, these studies provided information on immunoregulation by idiotype-antiidiotype interaction (11), on which future experiments in the human system might be based. Cross-reacting and dominant idiotypes have now been characterized for human anti–A-CHO antibodies (12). (*c*) A-CHO and its immunodominant hapten *N*-acetyl-D-glucosamine (GlcNAc) are structurally well characterized (13). Even the three-dimensional structure of A-CHO was recently revealed (D. Bundle, National Research Council, Ottawa, personal communication). (*d*) A large number of experiments, including studies on A-CHO–specific antibodies from man, mouse, and rabbits demonstrated a restricted clonal heterogeneity and a pronounced clonal dominance for this response (7, 8, 10); this situation may facilitate precursor frequency analyses of

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[1] *Abbreviations used in this paper:* A-CHO, streptococcal A carbohydrate; AET, 2-aminoethyl-isothiouronium bromide hydrobromide; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; MHC, major histocompatibility complex; PBL, peripheral blood lymphocyte; PWM, pokeweed mitogen; Strep A, streptococci group A; SUP, supernatant.

B cell clones. (*c*) The anti–A-CHO response requires cooperation with regulatory T cells (14).

We report here that (*a*) in sera of randomly selected, healthy human individuals without anamnestic rheumatic fever, proportions of >10% A-CHO–binding IgM or IgG are found that are mainly directed to GlcNAc. (*b*) Antibodies produced in vitro are predominantly of the IgM isotype, independent of whether they are produced by polyclonal or by antigen-specific activation. This is in contrast to the anti–A-CHO antibodies produced in vivo, and to in vitro–produced antibodies of other specificities that contain substantial proportions of IgG. Therefore, anti–A-CHO antibodies appear to be produced by B cells that may represent a functionally distinct subpopulation. (*c*) The proportions of IgM anti–A-CHO found in serum and in activated macrocultures are paralleled by equivalent B cell precursor frequencies for IgM anti–A-CHO in peripheral blood lymphocytes (PBL), suggesting that the IgM-producing B cell precursors are equally frequent in peripheral blood and in the antibody-producing lymphoid tissues, with a mean IgM production rate not exceeding the mean of the entire B cell pool.

Materials and Methods

Cell Separations. Human peripheral blood mononuclear cells from healthy volunteers were obtained from Ficoll/metrizoate density gradients (1,077 g/cm^3) in a standard procedure (15). B cells were prepared by rosetting T cells with 2-aminoethylisothiouronium bromide hydrobromide (AET) (Sigma Chemical Co., St. Louis, MO)–treated sheep red blood cells followed by a Ficoll/metrizoate gradient (16). The B cell–enriched population was collected from the interphase, containing 50–70% Ig$^+$ cells, <8% T3$^+$ cells, as revealed by peroxidase-antiperoxidase and fluoresence staining (cytofluorometric), and 20–40% monocytes positive for *o*-naphtyl-acetate-esterase. In the T cell–enriched population, sheep red blood cells were removed by hypotonic lysis with distilled water at 4°C for 20 s.

Antigen Preparation. For vaccine preparation, group Strep A, strain 17A4, were heat-killed and cleaved from their protein coat by pepsin treatment, as described (17). The concentration of organisms was adjusted by counting in a hemocytometer chamber. Group-specific carbohydrates of Strep A and C (strain C74) were prepared by $NaNO_2$/acetic acid extraction (E. C. Gotschlich, personal communication). Briefly, 8 g streptococci cultured in Todd-Hewitt broth were suspended in 80 ml Aqua bidest. 10 ml 4 NNO_2 and 10 ml acetic acid were added, and the suspension was stirred for 15 min. Detritus was centrifuged at 15,000 g_{max} for 10 min and the SUP (supernatant), containing the group-specific carbohydrate, was extensively dialysed against distilled water, and lyophilized. For coupling to plastic sites, the carbohydrates were esterified with stearic acid (18). The monosaccharides, GlcNAc and *N*-acetyl-D-galactosamine (GalNAc), used for inhibition studies, were purchased from Sigma Chemical Co.

Antibodies. Antibodies reactive to GlcNAc were isolated from human sera as described (19), by absorption on GlcNAc-agarose columns, and subsequently eluted by 5% GlcNAc. They were separated into the IgM and IgG fractions by high-pressure liquid chromatography on a TSK 3000 column (LKB Instruments, Inc., Gaithersburg MD).

$F(ab')_2$ and Fab' fragments were prepared as described (12). A goat antiserum specific for human γ, α, and μ chains was precipitated by ammonium sulfate and used in solid-phase enzyme-linked immunosorbent assays (ELISA). Reference sera adjusted to World Health Organization (WHO) standards for human IgM and IgG (Behringwerke, Marburg, Federal Republic of Germany [FRG]) were purchased. Goat anti–human IgM and goat anti–human IgG labeled with alkaline phosphatase were obtained from Medac (Hamburg, FRG).

Culture Conditions. Cultures for the determination of SUP Ig production were performed either in the original Mishell-Dutton conditions (20), with the modification of

using Dulbecco's modified Eagle's medium supplemented with 2-mercaptoethanol (10^{-5} M, final concentration), sodium pyruvate (0.11 g/liter) L-asparagine (0.036 g/liter), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS); or in Click's medium (21) supplemented with penicillin, streptomycin, and 10% FCS at 2% CO_2, without rocking.

Polyclonal and Antigen-specific In Vitro Activation of Human Lymphocytes. For polyclonal activation, 1.5×10^6 human PBL were cultured in 1-ml flat-bottom wells (Nunc GmbH, Wiesbaden, FRG) together with pokeweed mitogen (PWM) in a 1:200 final dilution of the stock solution (Gibco Laboratories, Grand Island, NY). Some experiments were carried out with cryopreserved human PBL. Previous cryopreservation did not alter the yield of antibodies produced in culture, nor the B cell frequencies determined by limiting dilution (F. Emmrich, unpublished observation). Antigen-specific activation was achieved by incubating 2×10^5 unseparated PBL per well together with heat-killed, pepsin-cleaved Strep A in 200-μl round-bottom microtiter wells (Nunc GmbH). The microorganisms were titrated as indicated. Culture supernatants were tested by solid-phase ELISA for various antibodies produced during the culture period. Antibodies appear in measurable amounts generally on day 4 and reach a plateau between days 8 and 12. By the cell separation procedure described above, no carry over of cytophilic Ig was observed that exceeded our sensitivity threshold of 100 pg/ml. Culture SUP harvested at day 12 by centrifugation were stored at −20°C in microtiter plates until assay.

Antibody Assays. Solid-phase ELISA were performed essentially as described (19) for the determination of anti–A-CHO antibodies as well as for the determination of total IgM and IgG in cell culture SUP. Human IgM anti–A-CHO or IgG anti–A-CHO reference preparations of a human serum pool (10 sera of healthy blood donors) were diluted in culture medium, and a seven-point duplicate standard dilution curve, ranging from 0.26 to 4,000 ng/ml, was set up on each assay plate. In general, plates were measured after 30 min, 2 h, and 24 h by an automatic photometer (MR-580; Dynatech Laboratories, Inc., Alexandria, VA) in order to find high as well as low test values in sensitive regions of the shifting standard curve. Standard curves were calculated and drawn by an Applesoft BASIC program (SPLINE 4) which uses spline functions to find an optimal nonlinear standard curve for calculating Ig concentrations.

For inhibition studies, soluble carbohydrates or monosaccharides were added in various concentrations, as indicated in Fig. 1, to culture SUP containing anti–A-CHO antibodies, or to purified reference antibodies, before incubation of antigen-coated microtiter plates.

Determination of B Cell Precursor Frequencies. B cell precursor frequencies for IgM or IgM anti–A-CHO–producing cells were determined by limiting-dilution analysis of replicate microcultures. Varying numbers of AET-rosette–negative cells in $\log_2$ dilution steps were cultured on 10^5 unseparated or T cell–enriched PBL irradiated with 3,000 rad in 96-well round-bottom microtiter plates in a final volume of 0.2 ml Click's medium with 10% FCS containing PWM (1:200) as polyclonal activator. 20 replicate cultures were established for each dose of B cells, ranging from 2×10^5 to 40 cells in the experiments described. In culture wells with 2×10^5 and 1×10^5 cells, the amount of feeder cells was reduced to 5×10^4. As mentioned above, the rosette-negative population includes 30–50% non–B cells, so that the determined B cell frequencies are underestimated up to 100%. For control, five wells with no added B cells and five wells with 2×10^4 irradiated B cells were included in each culture plate, containing, in general, three culture groups. After 12 d the culture SUP were harvested and stored until antibody assay. Those wells with more Ig than the arithmetic mean plus three standard deviations of the controls were scored as "positive", and the fraction of negative wells was calculated for each B cell dose.

Poisson analysis was used to determine the precursor frequency for Ig-producing cells (for theory, see reference 22) by plotting the number of cells against the proportion of negative wells within each test group. Frequencies were calculated by an iterative program (MINCHI), written in Applesoft, which provides four mathematically different fitting approaches. The minimum χ^2 method was selected according to Taswell (23), considering his criteria that beside $P > 0.05$, the frequency determined by minimum χ^2 should stay within ±15% of that determined by the maximum likelihood method.

TABLE I

A-CHO–specific Antibodies and Total Ig in Human Sera of Individual Donors and in Culture SUP of Their Polyclonally Activated Lymphocytes

Material	Donor	Ig concentration*			
		IgM	IgG	IgM anti-A-CHO	IgG anti-A-CHO
			μg/ml		
Serum or plasma	HZO	1,400	8,020	77	760
	HTA	3,800	17,600	55	226
	MSS	2,540	11,900	332	230
	BGI	1,340	8,010	18	157
	CMR	2,200	1,130	20	6
	JEN	1,700	7,200	26	71
	RRA	2,000	9,080	36	78
	BMR	1,500	13,100	91	590
			ng/ml		
Culture supernatant‡	HZO	4,660	10,109	1,362	62
	HTA	5,092	6,068	183	20
	MSS	6,290	12,222	978	15
	BGI	1,827	2,607	35	2
	CMR	3,825	1,367	397	1
	JEN	3,297	1,558	16	2
	RRA	1,589	1,455	65	4
	BMR	15,835	15,750	1,722	6

* IgM and IgG in serum or plasma were determined by radial immunodiffusion using WHO-proved standards. IgM and IgG in culture SUP were determined in solid-phase ELISA by means of the same standards. IgM anti-A-CHO and IgG anti-A-CHO were measured in serum as well as in SUP by ELISA using as standards affinity-purified, HPLC-separated preparations of specific antibodies diluted in culture medium containing FCS.

‡ Polyclonal activation was carried out in 1-ml flat-bottom wells by culturing 1.5×10^6 mononuclear lymphocytes with PWM (1:200) for 12 d in Click's medium with 10% FCS at 2% CO_2.

Results

Quantities of Total IgM and IgG, and of A-CHO–specific IgM and IgG Produced In Vivo and by Polyclonal Activation In Vitro. We studied seven randomly chosen healthy adults in these experiments, as well as one donor (MSS) specially selected because of a major GlcNAc-binding antibody spectrotype in his serum (12). We determined IgM and IgG concentrations by radial immunodiffusion, whereas we measured IgM anti-A-CHO and IgG anti-A-CHO concentrations by a solid-phase ELISA. For comparison, PBL of these donors were stimulated in vitro by PWM, and after 12 d, total Ig and specific Ig were measured in the cell culture, also by solid-phase ELISA. A compilation of these results appears in Table I. We prepared the reference antibodies used as standards for the ELISA assay from a pool of 10 human sera; they represent average avidity spectra as judged by comparing their binding curves with that of individual antibody preparations (data not shown). Strikingly high proportions of total IgM and IgG of all donors bound to A-CHO, ranging from 1.0 to 13.1% for IgM anti-A-CHO and from 0.5 to 9.5% for IgG anti-A-CHO in serum. In culture SUP, we found high proportions of anti-A-CHO (0.5–29%) for IgM but not for IgG (0.07–0.6%)

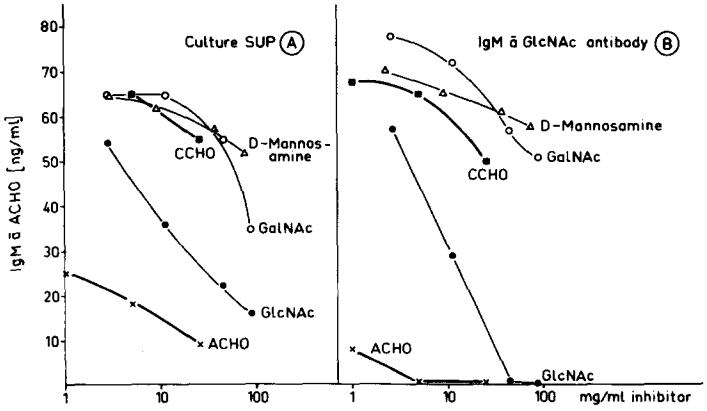

FIGURE 1. Fine specificity analysis of anti–A-CHO produced after polyclonal B cell activation in vitro (*A*) compared with affinity-purified anti-GlcNAc IgM adjusted in culture medium to the same antibody concentration (*B*). IgM anti–A-CHO was measured by a solid-phase ELISA using stearoyl–A-CHO as antigen, and an enzyme-labeled anti–human IgM as detection reagent.

(Table I). The ratio of IgM anti–A-CHO to IgG anti–A-CHO ranged from 0.1 (donor HZO) to 3.3 (donor CMR) in serum, whereas, in strong contrast, in vitro polyclonally activated PBL produced mainly IgM anti–A-CHO with IgM/IgG ratios of 8 (JEN) and 397 (CMR) to one. The deficiency in IgG anti–A-CHO production was not a consequence of a deficiency in the total IgG production in vitro, because total IgM/IgG ratios were, in most cases, only slightly lower than in serum.

Fine Specificity of Anti–A-CHO Antibodies Produced In Vitro. A-CHO–specific antibodies in sera of immunized animals (24) or of human donors (7) consist mainly of antibodies to GlcNAc, the immunodominant group of the A-CHO. To analyze the fine specificity of anti–A-CHO antibodies produced in vitro we used various mono- and polysaccharides. They were tested for their ability to inhibit the binding to solid-phase A-CHO of anti–A-CHO antibodies in culture SUP of polyclonally activated PBL (Fig. 1). This was compared with the affinity-purified IgM anti-GlcNAc preparation from the serum of the same individual, after adjusting to a similar Ig concentration as the antibody produced in vitro. A-CHO and its monosaccharide hapten GlcNAc inhibited both antibody populations, whereas we saw no inhibition with the carbohydrate of C-streptococci (C-CHO), which carries *N*-acetyl-D-galactosamine (GalNAc) disaccharide side chains instead of GlcNAc on the same rhamnose backbone (13). As usual, inhibitions with the monosaccharides required higher inhibitor concentrations than with the complete carbohydrate molecule. From these results, we conclude that, similar to antibodies produced in vivo, most anti–A-CHO antibodies produced in vitro are directed to GlcNAc.

Determination of B Cell Precursor Frequencies for IgM and IgM Anti–A-CHO. To study whether the high proportions of A-CHO–specific IgM in vivo and in vitro were reflected in PBL with an equally high proportion of B cells of the same specificity, we did the following experiments. We determined the frequencies of IgM-producing B cells and the frequencies of B cells that produce IgM anti–

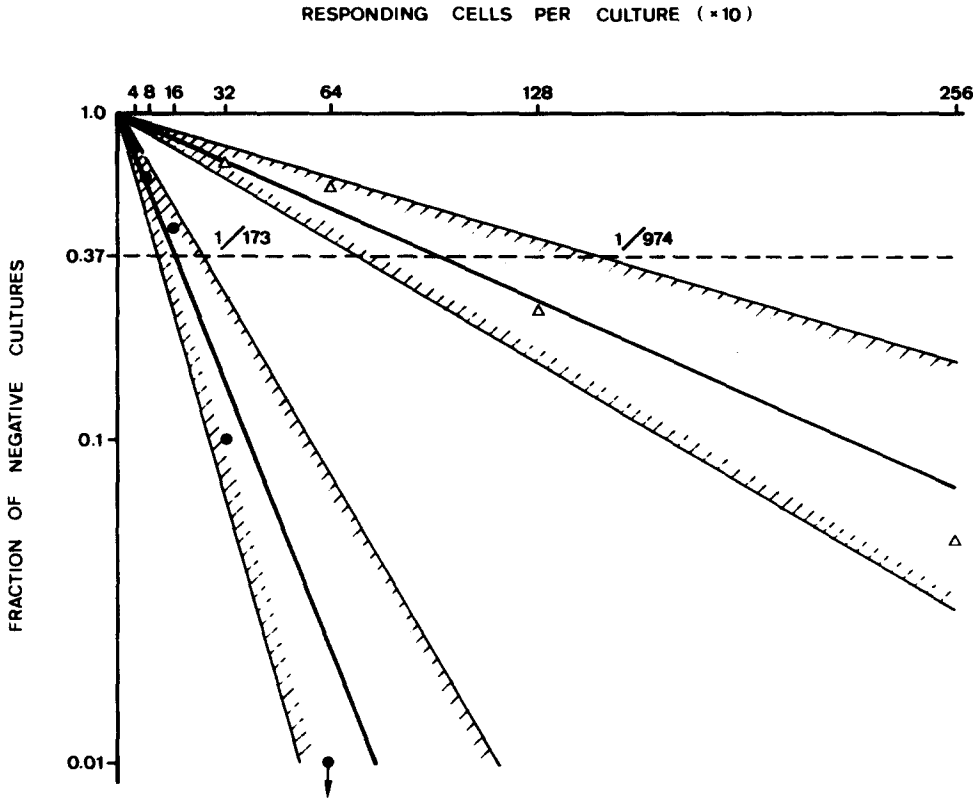

FIGURE 2. Precursor frequency analysis of Ig-producing cells after polyclonal activation. Human B cells from donor MSS were enriched by rosetting with AET-treated sheep red blood cells. The rosette-negative population was added in twofold dilutions (2×10^5–40 cells/culture) to round-bottom microtiter wells (20 wells/group) containing 10^5 irradiated autologous PBL and 1:200 PWM as polyclonal activator. After 12 d, culture SUPs were collected and measured for IgM (●——●) and for anti-A-CHO IgM (△——△). Mean Ig values plus three SD of cultures with 2×10^4 irradiated B cells were used as thresholds for negative cultures. Frequencies of antibody-producing cells and SD were calculated according to Poisson statistics by a computer program on the base of the minimum χ^2 procedure. The hatched area refers to the 95% confidence interval.

A-CHO by limiting dilution analyses using B cell–enriched PBL. We chose three donors (MSS, HTA, JEN) with high, intermediate, and low proportions, respectively, of IgM anti-A-CHO antibodies after polyclonal activation in macrocultures (see Table I). Fig. 2 shows a representative experiment. By plotting cell numbers versus the log of the fraction of negative cultures, we obtained straight lines in all cases, demonstrating single-hit kinetics, which allows the calculation of precursor frequencies (22). All frequencies are minimum estimates because of the contamination by non-B cells. In order to obtain correct ratios, we adjusted the detection limits of the assays (used for both IgM and IgM anti-A-CHO) to equivalent sensitivities. Proportions of IgM anti-A-CHO–specific B cells (absolute frequencies) were calculated by dividing their precursor frequencies by that of IgM-producing cells.

Table II gives B cell precursor frequencies for IgM and for IgM anti-A-CHO

TABLE II

Determination of B Cell Precursor Frequencies for IgM and IgM Anti-A-CHO After Polyclonal Activation With PWM

Donor		Precursor frequency*				IgM anti-A-CHO/IgM ratio		
		IgM		IgM anti-A-CHO		Φ‡	Macroculture§	Serum‖
						%		
MSS	Exp. 1	1:4,272	(1:3,137–1:6,693)	1:22,727	(1:16,393–1:37,037)	18.8	15.5	13.1
	Exp. 2¶	1:173	(1:129–1:266)	1:974	(1:732–1:1,456)	17.8		
HTA		1:279	(1:186–1:564)	1:6,361	(1:4,744–1:9,653)	4.4	3.6	1.4
JEN		1:119	(1:90–1:176)	1:37,175	(1:13,072–1:51,546)	0.3	0.5	1.5

* Results in parentheses limit the 95% confidence intervals.

‡ Percentages calculated from the precursor frequencies in PWM-activated microcultures, using the results from this table.

§ Percentages calculated from the IgM anti-A-CHO and IgM concentrations of PWM-activated macrocultures given in Table I.

‖ Percentages calculated from serum Ig concentrations given in Table I.

¶ Exp. 2 of donor MSS is shown in Fig. 1. For experimental details see legend of Fig. 1.

from donors MSS, HTA, and JEN. For comparison, ratios of soluble antibodies, calculated from polyclonally activated macrocultures and from antibody determinations in serum, are included. Within the reproducibility of the assays, we obtained the same proportions from all three calculations, for each donor. This suggests that (*a*) B cell precursors for IgM anti-A-CHO are nearly equally frequent in PBL and in the lymphoid tissues; and (*b*) the mean IgM anti-A-CHO production per B cell is similar to the mean IgM production of all B cells in vitro.

Two of five experiments for donor MSS are shown in Table II, representing the highest (1:173) and lowest (1:4272) frequency of IgM-producing cells. This pronounced experimental variability is primarily due to variation in the efficiency of PWM activation. In spite of this variability, we found the same absolute frequencies of IgM anti-A-CHO–producing B cells in the experiments with the lowest and highest efficiency of mitogen activation.

In agreement with other groups, <1% of all human peripheral blood B cells are activated by PWM (25, 26), in contrast to mouse B cells, of which 30% may be activated polyclonally (27). In view of the low percentages of B cells activated by PWM, and the high proportion of A-CHO–specific B cells among them, we suspected that PWM preferentially stimulated A-CHO–specific B cells. We determined IgM anti-A-CHO/total IgM ratios, using different polyclonal activation conditions. We had previously found that, under Mishell-Dutton conditions at high cell densities (10^6 cells per round-bottom microtiter well), spontaneous polyclonal activation occurs, resulting in IgM production >20-fold greater than that of the same number of PBL cultured in the same volume, with the same FCS, in flat-bottom wells. PWM does not substantially increase IgM production when added to such high-density cultures. For cells from donor MSS, the proportions of IgM produced by high-density activation that had specificity to A-CHO ranged from 6.2 to 33.3% (mean, 15.8%) in six independent cultures, and thus are similar to the data shown in Table II for PWM activation of PBL from MSS. Preferential PWM activation of IgM anti-A-CHO–producing B cells is therefore unlikely. However, in cultures without antigen or mitogen, at low cell densities, the proportion of IgM anti-A-CHO is very much smaller than in

TABLE III

IgM Anti–A-CHO Proportions in Cultures With Varying Cell Density and Different Activation Conditions

PBL ($\times 10^5$)	Activation	Culture well	IgM	IgM anti–A-CHO	IgM anti–A-CHO
			ng/ml	*ng/ml*	%
1.5	—	F	507 ± 35	5 ± 4	1.0
1.5	Strep A	F	480 ± 56	78 ± 30	16.3
2	—	R	1,020 ± 220	25 ± 9	2.5
2	Strep A	R	1,330 ± 370	200 ± 170	15.0
10	—	R	4,508 ± 1354	713 ± 512	15.8
2	PWM	F	6,200 ± 852	775 ± 334	12.5

Varying numbers of PBL (donor MSS) were cultured for 12 d with 10% FCS in either round-bottom (R) or in flat-bottom (F) microculture wells (200 μl) with mitogen (PWM 1:200) or antigen (Strep A), or without an addition. IgM and IgM anti–A-CHO were determined by solid-phase ELISA.

cultures with either high density, or mitogen- or antigen-induced activation. This is shown in Table III for donor MSS, and in the control cultures of Table IV for donor CMR. We think that under these in vitro conditions, only those B cells that had been preactivated in vivo by unknown antigens secrete IgM. Alternatively, B cells specific for FCS components might be preferentially stimulated under these culture conditions.

We also considered the remote possibility that A-CHO binding occurs via the constant part of IgM, particularly in the case of donor MSS whose IgM contained 15% A-CHO–binding antibodies. However, using Fab' fragments of affinity-purified IgM anti-GlcNAc antibodies from that donor, we found binding to solid-phase A-CHO at levels similar to those seen with the complete molecule. Both antibody preparations bound specifically to A-CHO only, and not to the closely related carbohydrate of group C streptococci (data not shown).

Induction of a Specific In Vitro Response to Strep A Carbohydrate. Among a variety of culture systems, we found that the original Mishell-Dutton procedure (20) with Mishell-Dutton medium, rocking platform, and the special gas mixture (10% CO_2, 7% O_2, 83% N_2), and the Click's system (21), provided adequate conditions for the specific induction of anti–A-CHO antibodies. Specific responses could be induced only by the bacterial particles, and not by the soluble A-carbohydrate (data not shown). Fig. 3 shows IgM anti–A-CHO antibody concentrations among PBL from five donors, stimulated by various doses of Strep A vaccine. Only in these donors (CMR, MSS, and HTA) could anti–A-CHO antibody secretion be enhanced, with a peak response at 2×10^3 to 2×10^4 streptococcal particles per microculture. It should be noted that values of individual culture wells varied far more than observed in PWM-stimulated cultures. This may indicate that, for specific activation, spatial relationships between cooperating cells might be important. In some donors, especially in donor MSS, IgM anti–A-CHO antibodies were spontaneously produced to a considerable extent. This phenomenon was not caused by cytophilic antibodies, because cultures harvested immediately after being established were consistently negative for anti–A-CHO antibodies.

TABLE IV

Proportions of A-CHO–specific Antibodies and Total Ig in Individual Microcultures of PBL Cultured With Various Quantities of Enzyme-cleaved Strep A

Strep A/culture	Antibody	Ig concentration in well:			
		A	B	C	D
		ng/ml			
	IgM	2,600	1,050	1,900	1,050
	IgG	800	140	250	380
—	IgM anti-A-CHO	4	3	8	5
	IgG anti-A-CHO	*	—	1	*
	IgM anti-C-CHO	6	6	12	7
		2,200	900	12,200	500
		600	210	2,600	190
2×10^2		160	8	*521*	302
		4	*	4	—
		6	6	400	21
		2,500	2,800	18,000	1,200
		360	500	8,000	270
2×10^3		92	*776*	299	105
		*	—	11	*
		5	21	134	18
		16,000	1,350	18,100	3,000
		1,500	300	6,300	1,200
2×10^4		*4,868*	*1,265*	*3,422*	*1,042*
		8	*	6	*
		17	10	400	56
		460	2,050	4,200	670
		80	360	2,600	120
2×10^5		4	210	18	34
		*	*	1	*
		3	40	9	4
		1,500	2,000	2,100	980
		240	290	980	110
2×10^6		8	5	15	4
		*	1	1	*
		11	7	18	3
		16,500	4,200	8,200	1,450
		44,000	800	4,200	460
2×10^7		36	13	14	7
		*	1	2	2
		1	10	19	6

2×10^5 PBL (from donor CMR) were cultured for 12 d in round-bottom microtiter wells with or without Strep A ranging from 2×10^2 to 2×10^7 Strep A particles per culture. Concentrations of specific antibodies and total IgM and IgG were determined by ELISA using 40 μl undiluted culture supernatants for the measurement of specific antibodies and 1:10 dilutions for IgM and IgG. A, B, C, and D represent single culture wells. IgM anti-A-CHO concentrations above 500 ng/ml are italic. Ig concentrations within the bending portion of the standard curve are represented by: (*) 0.3–1.0 ng/ml; (—) <0.3 ng/ml.

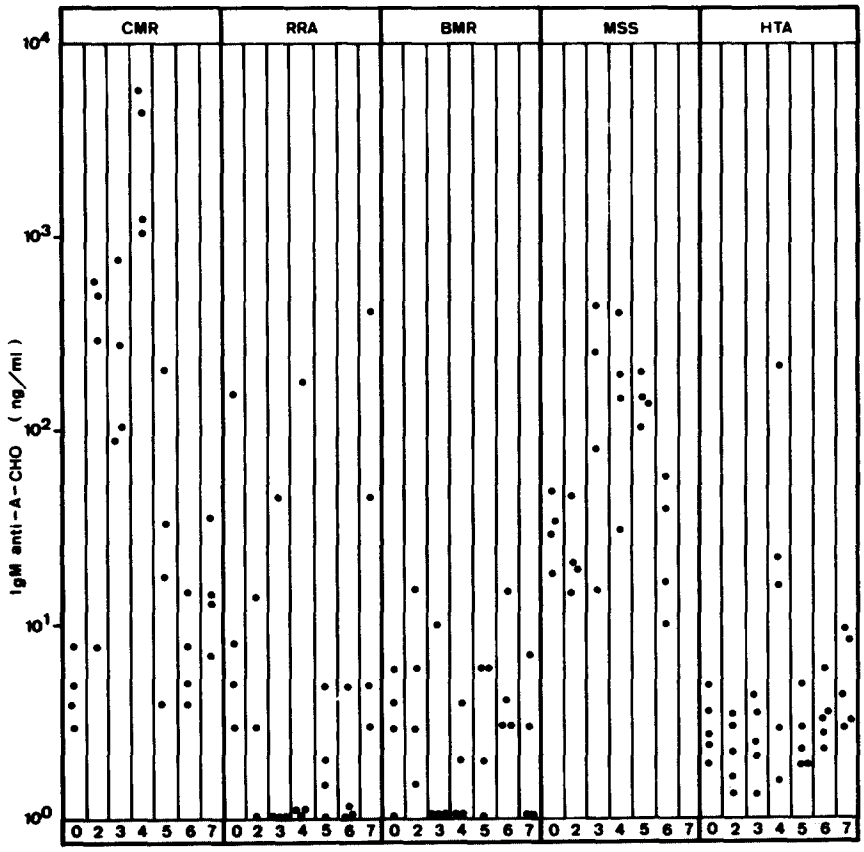

FIGURE 3. Anti-A-CHO IgM concentrations determined after 12 d in cultures (Click's medium, 10% FCS, round-bottom microtiter wells) of PBL from five different donors, incubated with various amounts of Strep A per culture: medium control (*0*), 2×10^2 (*2*), 2×10^3 (*3*), 2×10^4 (*4*), 2×10^5 (*5*), 2×10^6 (*6*), 2×10^7 (*7*). Each dot represents a single microculture well.

To demonstrate that the increase of anti-A-CHO antibodies was not a part of a polyclonal increase in total IgM, we performed the experiment shown in Table IV. Concentrations of various antibody populations were determined independently in cultures stimulated with six different streptococcal concentrations. Production of A-CHO-specific IgM was stimulated best at 2×10^4 Strep A per culture. Elevated total IgM was found in two cultures of this group, whereas the other two cultures showed normal IgM levels. The mean percentages of IgM anti-A-CHO were 0.33 ± 0.13% in the medium controls, and 21.3 ± 13.1% at 2×10^4 Strep A cells/well, corresponding to an increase in specific antibody ~60-fold greater than that of total IgM. Independent of the considerable amount of IgG produced in most wells, nearly no IgG anti-A-CHO was found, which is in agreement with the results of polyclonal activation. Not consistently, but in some wells of the groups at 2×10^2 to 2×10^4 Strep A cells/well, IgM antibodies against streptococcal C carbohydrate were also found.

At high antigen concentrations (>2×10^6 Strep A cells/culture), only low IgM

anti–A-CHO production was observed. The possibility that large amounts of streptococci in the culture could absorb the specific antibody was excluded by a 4-d pulse immunization of cultures, with subsequent removal of the bulk of Strep A. After an additional 8 d of culture, a similar depression of the responses was observed for cultures that had been pulsed with high amounts of streptococci, and in control cultures in which the streptococci were present for the whole culture period (data not shown). In contrast, as demonstrated in the last row of Table IV (culture A), total IgM and IgG were sometimes elevated in cultures containing high amounts of Strep A ($>2 \times 10^7$ Strep A cells/culture), suggesting a moderate polyclonal activation by Strep A vaccine at high concentrations.

Discussion

In recent studies on quantitative determinations of total anti–A-CHO antibodies in human sera (in which no distinction was made between IgM and IgG), concentrations of 20–250 μg/ml anti–A-CHO were reported for 30 randomly selected healthy donors (28). In contrast, in a group of 12 patients suffering from acute rheumatic fever, 70–660 μg/ml anti–A-CHO (29) were found. These studies used anti–A-CHO from hyperimmunized rabbits as reference antibody for quantitative determinations, measured in a fluid-phase radioimmunoassay. Because of a possibly higher average affinity of the oligoclonal rabbit antibodies, and because low-affinity human antibodies might be missed in the fluid-phase assay, the serum concentrations of anti–A-CHO, which are presumably of low average affinity, might have been underestimated in those studies. This might also explain why no IgM anti–A-CHO could be detected in human sera in a previously published survey (7).

To circumvent these problems, we used human IgM and IgG that had been taken from pooled serum and affinity-purified on A-CHO columns as reference for solid-phase ELISA measurement of specific antibodies. In eight healthy donors we found 26–837 μg/ml total anti–A-CHO. The accuracy of the results was confirmed by independent experiments. For instance, we could elute amounts of anti–A-CHO from A-CHO affinity columns as were predicted from serum determinations. This eluant was immunoelectrophoretically pure IgM and IgG (data not shown). Furthermore, for IgM, the proportions of anti–A-CHO in serum titers were reflected in the proportions of precursor B cells found in limiting dilution analyses (Table II). Therefore, proportions of A-CHO–specific antibodies comprising >10% of the total IgM or IgG appear not to be unusual even in healthy human individuals without a history of anamnestic rheumatic fever or frequent tonsillitis.

These antibodies are mainly of GlcNAc specificity and are restricted predominantly to the IgG2/K isotype (7). Anti-GlcNAc antibodies may have been induced not only by A-CHO, but also by other bacterial polysaccharides containing terminal GlcNAc moieties in a 1,3-glycosidic linkage, such as the O substance of gram-negative bacteria (30). Moreover, terminal GlcNAc is exposed transiently during synthesis or degradation of blood group substance (P. Hanfland, personal communication) and might act as an antigenic stimulus. We think, however, that the functional significance of the large amounts of anti-GlcNAc antibodies in healthy individuals lies in the persistance of streptococci in human tonsils without

any symptoms of tonsillitis. It is possible that the immune system prevents an overgrowth of these bacteria by maintaining a potent state of natural immunity. Varying antibody levels in individual donors may be due to genetic control, as demonstrated for rabbit (31, 32) and mice (33), as well as different levels of continuous challenge by the bacteria.

All human adults seem to be primed to Strep A, as revealed by an analysis of sera from ~200 healthy blood donors that contained IgM anti–A-CHO in varying amounts. Two cord-blood sera, on the other hand, showed a total lack of IgM anti–A-CHO, while containing IgG anti–A-CHO, presumably from the mother. In addition, we could not induce IgM anti–A-CHO secretion by polyclonal activation (PWM) of human cord-blood lymphocytes (F. Emmrich, unpublished experiments). Together, both experiments indicate the lack of priming in the newborn, and an acquired state of natural immunity in adults, as corroborated by previous experiments (5).

In view of the large amounts of anti–A-CHO antibodies in sera, it seemed interesting to evaluate, for comparison, the frequency of specific precursor B cells in PBL after polyclonal activation by PWM. Essentially similar proportions of IgM anti–A-CHO within total IgM were found in serum, SUP of macrocultures, and, by limiting dilution analyses of B cell precursors, in human peripheral blood, the latter amounting to 1/6–1/300 of all IgM-producing B cells. These results suggest a similar distribution of those B cells in lymphoid tissues and in peripheral blood, and also a similar mean IgM production rate when compared with the entire B cell pool.

Using well-established culture conditions, we could induce lymphocytes of some adult individuals to give an A-CHO–specific response. This response is somewhat erratic, and shows large variations between identical culture wells, which, in our hands, is not the case for in vitro secondary responses to tetanus toxoid (a model for a soluble protein antigen). This suggests rather limited requirements for the induction of an anti–A-CHO response that are not only dependent on the number of available precursor B cells (see donor BMR, Table II and Fig. 3). At high vaccine concentration ($>10^6$ Strep A cells/culture), diminished specific IgM anti–A-CHO levels were observed. This phenomenon was not due to absorption of the anti–A-CHO by large amounts of streptococci (see comment to Table IV), and may therefore reflect a specific suppression that is also observed in secondary in vitro responses to high antigen doses of soluble protein antigens (34, 35). Taken together, we think that not all of the regulatory factors that influence a specific anti–A-CHO response in vitro are sufficiently understood.

In PBL obtained from Strep A–immunized rabbits, IgM anti–A-CHO, as well as IgG anti–A-CHO, plaque-forming cells were induced in an in vitro secondary response, with the latter reaching 83% of the antigen-specific plaques after 15 d of culture (36). Concomitant secretion of IgG anti–A-CHO into the culture medium was shown. In contrast, we found nearly exclusively anti–A-CHO of the IgM isotype, in both antigen-activated or mitogen-activated human PBL cultures. Although slightly less IgG-secreting B cells are activated in vitro than expected from the IgM/IgG isotype proportion in serum (see Table I), total IgG in polyclonally activated cultures amounts to nearly the same as total IgM. There-

fore, the selective lack of IgG anti–A-CHO in vitro may suggest distinct differentiation requirements for anti–A-CHO–producing B cells compared with those B cell species which readily produce IgG in vitro.

As an alternative, instead of lacking in vitro differentiation, anti–A-CHO IgG–producing B cells are, perhaps, sequestered in certain lymphoid tissues. If this is the correct explanation of our results, we would have to conclude again that the switch from IgM to IgG production does not occur with human PBL under our culture conditions. Indeed, with two different polyclonal activators (PWM and Epstein-Barr virus) as well as with antigenic stimulation of human PBL, virtually no or very rare switch events from IgM to IgG could be demonstrated in vitro (25, 37). This is in sharp contrast to similar experiments in lipopolysaccharide-stimulated murine spleen cells (38). As discussed by Yarchoan et al. (25), either an intrinsic difference between human and murine B cells or a difference between peripheral blood B cells and those of other lymphoid organs might explain the results. Preliminary results with polyclonally activated human spleen cells and with tonsilar lymphocytes showed only slightly increased levels of anti–A-CHO IgG (F. Emmrich, unpublished data).

In the control cultures of donor CMR (data not shown) without mitogen or streptococcal antigen, the IgM anti–A-CHO ratio (0.33%) was far less than in both antigen-activated (21.3%) or PWM-activated cultures (10.3%, see Table I). This indicates preferential antibody production in the control cultures by non–anti–A-CHO B cells either preactivated in vivo or stimulated by FCS components in vitro. As demonstrated in Table III, low IgM anti–A-CHO proportions could be elevated by either antigen activation, mitogen activation, or even by increasing the cell density. By these procedures, IgM anti–A-CHO proportions of ~15% were reached, which are equal to the precursor frequency of polyclonally activated B cells, as well as to the IgM anti–A-CHO proportion in serum of donor MSS. Together with the other features mentioned above, this finding may argue for distinct activation requirements of IgM anti–A-CHO B cells, compared with other B cells. A similar situation has previously been described for mice: unresponsiveness to polysaccharide antigens, determined by the *xid* gene (39), is associated with the lack of a B cell subpopulation bearing the Lyb-5 marker (40), as well as depressed IgM and IgG3 levels (41). Lyb-5$^+$ B cells are readily responsive to the combination of antigen and soluble helper factors, whereas Lyb-5$^-$ cells require an MHC-restricted interaction with helper T cells (42). Hence, human B cells with specificity to A-CHO may be useful for the characterization of a similar human B cell subpopulation.

Summary

The immune response to the group-specific carbohydrate of group A streptococci (A-CHO) provides an informative in vitro model for the investigation of several aspects of human anticarbohydrate immune responses. A-CHO–specific B cells can be polyclonally activated by pokeweed mitogen (PWM), and, specifically, by in vitro immunization with streptococcal vaccine. High levels of A-CHO–specific antibodies, mainly directed to the immunodominant side chain *N*-acetyl-D-glucosamine (GlcNAc), occur in healthy adult individuals. Serum antibody levels are reflected in high frequencies of precursor B cells among periph-

eral blood lymphocytes. In one particular case, >15% of all B cells activated by PWM for IgM production were found to produce IgM anti–A-CHO antibodies, as determined in limiting dilution experiments, as well as by analyzing Ig concentrations in bulk culture experiments. The case with the lowest proportion observed had 0.3% A-CHO–specific B cells among IgM-producing B cells. Preferential PWM activation of anti–A-CHO–producing B cells could be excluded.

The comparison of the proportions of anti–A-CHO IgM produced in vivo, and of B cells producing antibodies of this specificity in peripheral blood, suggests a similar distribution of specific precursor B cells in the antibody-producing lymphoid tissue compartments and in peripheral blood. However, nearly all specific antibodies produced in vitro belong to the IgM isotype, whereas IgG anti–A-CHO in high amounts, mostly exceeding the specific IgM, was found only among anti–A-CHO antibodies produced in vivo. Low anti–A-CHO IgG production was seen in polyclonally activated as well as in antigen-activated cultures, whereas, in contrast, total IgG was produced in considerable amounts after polyclonal activation. This suggests a different distribution pattern, and/or diverse differentiation requirements for anti–A-CHO–producing B cells, compared with other B cell species.

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