ANTIBODY RESPONSE OF RABBIT BLOOD LYMPHOCYTES IN VITRO

Kinetics, Clone Size, and Clonotype Analysis in Response to Streptococcal Group Polysaccharide Antigens

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Hyperimmunization of rabbits and mice against streptococcal groups A-variant and A polysaccharides (Av-CHO and A-CHO)[1] leads to the induction of a long-lived immunological memory. The number of clonotypes of specific IgG antibodies remains constant with time and repeated immunizations. This pattern is already stable from the moment when IgG antibodies are first detectable (1).

It was previously shown that peripheral blood lymphocytes (PBL) from rabbits primed in vivo with the Av-CHO can be restimulated in vitro. It was observed that the pattern of IgG antibodies overlapped with the clonotype patterns induced in vivo (2). In the present paper the state of immunological memory was investigated by using both conventional and microculture techniques (2–4).

Materials and Methods

Rabbits. The rabbits used within this study were taken from a colony bred selectively for restricted high responders (5). They had been previously immunized with dead vaccines of Group A-variant (strain A486 variant, M-) streptococci and produced 10–25 mg/ml of antibody at the peak of the response. Rabbit K19-205 was additionally immunized with a Group A (strain J17A4) streptococcal vaccine 6 mo after the injection course with the Group A-variant vaccine.

Cultures. Rabbit blood was collected from the central artery of the ear into heparinized bottles. The erythrocytes were sedimented in 1% pig skin gelatin, as described by Sell and Gell (6), and the leukocytes were washed and resuspended in RPMI 1640 medium (Microbiological Associates, Bethesda, Md.) containing 10% fetal calf serum (Rehatuin, Reheis Chemical Co., Chicago, Ill., Batch No. H73212), 5×10^{-5} M 2-mercaptoethanol, and 50 U/ml of both penicillin and streptomycin (Microbiological Associates). Streptococcal A-variant or A vaccines were added as antigen in doses of 2.5-3 μg rhamnose per ml (2). 1-ml cultures containing 4×10^6 or 2×10^6 cells were distributed in plastic tubes (Falcon Plastics, Los Angeles, Calif., type 2003). For microcul-

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[1] *Abbreviations used in this paper:* A-CHO, streptococcal group A polysaccharide; Av-CHO, streptococcal group A variant polysaccharide; [^{125}I]A-CHO tyr, [^{125}I]Av-CHO tyr, [^{131}I]Av-CHO tyr, isolated, labeled polysaccharides which were first tyraminated and then labelled with ^{125}I or ^{131}I; A-CHO-O-St and Av-CHO-O-St, O-stearic acid esters of the isolated polysaccharides; IEF, isolectric focusing; PBL, peripheral blood lymphocytes; PFC, plaque-forming cells; SII, pneumococcal type II polysaccharide; SRBC, sheep red blood cells.

tures 10^4, 2×10^4, 4×10^4, or 8×10^4 cells were distributed in 10-μl aliquots in microculture trays (Falcon Plastics, type 3034) as previously described (3–4). Both 1-ml cultures and microcultures were kept in a 5% CO_2 incubator at 37°C without further treatment during the culture period.

Reagents to Identify Specific Antibodies. The isolated, chemically pure A-CHO and Av-CHO (7–9) were used in derivatized forms. For labeling with the isoptopes ^{125}I and ^{131}I a prior tyramination was required to prepare the following reagents reactive with specific antibody: [^{131}I]A-CHO tyr, [^{125}I]Av-CHO tyr (10). For coating of sheep red blood cells (SRBC) the O-stearoyl esters of the A-CHO and the Av-CHO were used (2). Both forms of SRBC were strongly agglutinated by specific antibodies and specific lysis occurred upon the addition of complement.

Assay Systems to Measure Antibody Production. Production of specific antibody to the A-CHO and Av-CHO was scored by enumeration of plaque-forming cells (PFC) and identification of antibody in culture supernates. Specific PFC in 1-ml cultures and microcultures were identified using the method of hemolysis in agar, as originally described by Jerne et al. (11), and modified for microscope slides by Mishell and Dutton (12). The indicator systems were SRBC coated with A-CHO and Av-CHO antigens (A-CHO-O-St-and Av-CHO-O-St-SRBC) as previously described (2). Background PFC were determined with uncoated SRBC. The presence of antibody in the microcultures supernates was detected with the spot test (4) using Av-CHO-O-St-SRBC (2). In this case controls were performed also with noncoated SRBC.

Analysis of clonotypes produced in cultures was performed by analytical isoelectric focusing (IEF) followed by overlaying with [^{125}I]Av-CHO, washing, and autoradiography (5, 10). When single microculture supernates were assayed IEF analysis of high sensitivity, but lower resolving power, was used. This was done on gels reduced in length to one-third of the regular polyacrylamide thin-layer gels. Pools of microculture supernates and supernates from 1-ml cultures were assayed on regular IEF plates (5,10). For this, pools of microculture supernates and supernates of 1-ml cultures were concentrated 20–50-fold.

In some experiments performed with PBL of rabbit K19-205 (1-ml cultures) the synthesized antibody was quantitated by a modified Farr assay using [^{125}I]A-CHO tyr and [^{125}I]Av-CHO tyr (10). The specific immune sera of this rabbit were used as references.

Results

Antibody Response in 1-ml Cultures

INDUCTION OF PFC. PBL from five different rabbits that had received in vivo one or two immunization courses with the Av-CHO vaccine were stimulated in culture with this antigen at a cell concentration of 2×10^6 or 4×10^6 per ml, and the PFC response was followed over a period of 15–20 days starting at day 5. In agreement with previous data (2) a good response was consistently observed, with a maximum between days 10–20. The recovery of viable cells in all these experiments is given in Table I. In a later set of experiments rabbit K19-205, which had previously received immunization courses with three different bacterial vaccines (SII, Av-CHO, A-CHO), was bled at different intervals over a period of 4 mo. The PBL were stimulated in culture with Av-CHO and A-CHO vaccines. Fig. 1 shows the kinetics of the PFC response to Av-CHO and A-CHO. The onset of the anti-Av-CHO response was consistently faster than that of the anti-A-CHO response; while the response at its maximum was similar for the two antigens. Once the maximum was reached no apparent drop of the response was observed until day 42; in the second experiment this was found to be true up to day 55 (not shown in the figures). Note that in all three experiments the same pattern of the response with both antigens was obtained.

AMOUNT OF SECRETED ANTIBODY. An attempt was made to measure the amount of antibody produced in culture. The results for the anti-Av-CHO response are given in Fig. 2.

TABLE I
Recovery of Viable Cells During In Vitro Cultivation of Rabbit PBL

Cell input/culture	Antigen	Rabbit	Viable cells × 10^{-6}/culture at days:							
			5	7	10	14/15	20/21	28	42	55
		K9-246	0.38	ND[1]	0.38	0.2	0.12			
		K9-336	0.75	ND	0.29	0.13	ND			
4 × 10^6	Av-CHO	K31-143	0.35	ND	0.17	0.21	0.08		ND	
		K31-145	0.3	ND	0.05	0.08	0.08			
		K19-262	0.22	ND	0.21	0.2	ND			
	—		ND	0.49	ND	0.87	0.30	0.48	0.06	ND
2 × 10^6	A-CHO	K19-205	ND	1.06	ND	1.56	0.30	0.38	0.5	ND
	Av-CHO		ND	1.3	ND	0.83	0.46	0.28	0.24	0.32

[1] ND, not determined.

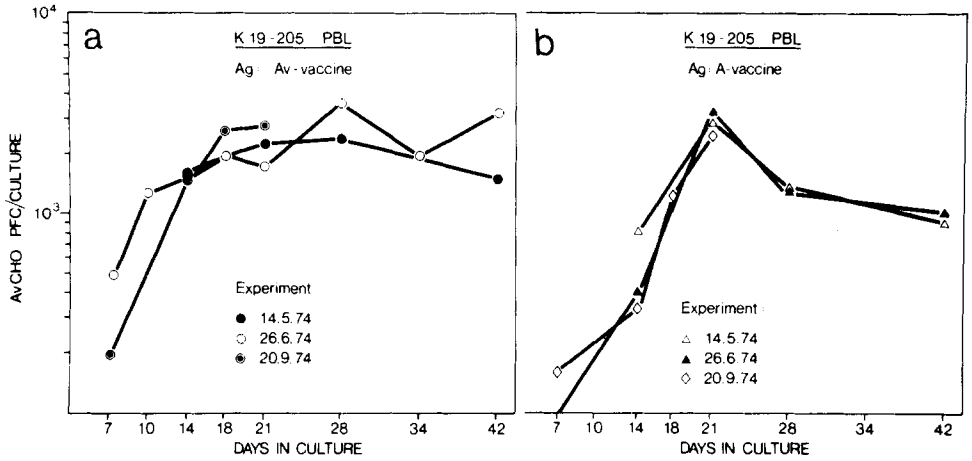

FIG. 1. PFC response of K19-205 PBL to Av-CHO (Fig. 1 a) and A-CHO (Fig. 1 b) in three different experiments. Experiment 26.6.74 with Av-CHO antigen in culture was run until day 55 (not shown here) at which time 1,500 PFC/culture were counted. Note the slower kinetics in the A-CHO response up to day 14 and the accelerated response until the peak of PFC was reached.

Anti-Av-CHO antibody became detectable by the Farr assay at day 10, at which time 2.5 μg/culture were found. In two of the experiments antibody concentrations continued to increase until day 34 when levels of around 40 μg/culture were found, and no further increase was observed after this time. Thus the peak of secreted antibody lagged behind the PFC response approximately 1 wk. Similar concentrations of specific antibody were reached for the anti-A-CHO response.

Antibody Response in Microcultures

PRECURSOR CELLS. The stimulation of single precursor cells can conveniently be analyzed by the microculture technique originally developed for the SRBC system (4,13–14). For estimation of the frequency of precursor cells a limiting dilution analysis should be used. For such a limiting dilution analysis it is necessary to culture graded numbers of lymphocytes to see whether the response

follows single hit kinetics. For the purpose of calculating the frequency of precursors, only such single hit kinetics can be interpreted unambiguously. As it was shown earlier, the cell density in rabbit PBL experiments can be varied only in a very narrow range (3). This earlier finding reported for anti-SRBC response also held for the anti-Av-CHO response (Fig. 3), indicating that the efficiency of the response is highly dependent on the cell density. In Fig. 3 a, kinetics of the response to Av-CHO is shown using four different cells densities. The optimal cell density for a response was 2×10^6/ml. The data shown in this figure are based on the spot test analysis of 120 microcultures for each experimental point. 1-ml cultures run in parallel agreed with these data in both the time of onset of the response and the optimal cell density.

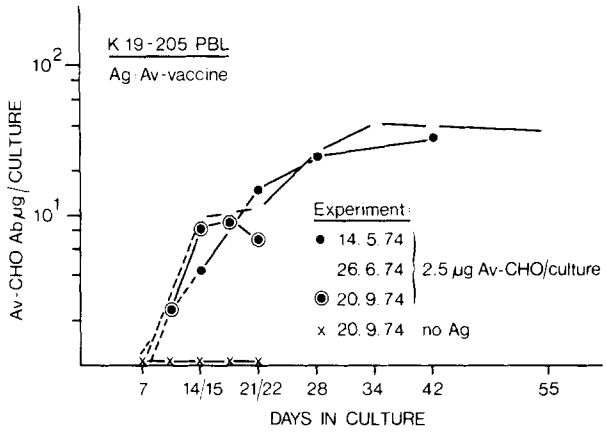

FIG. 2. Levels of free anti-Av-CHO antibody (Ab) in supernates of K19-205 PBL cultures stimulated by the Av-CHO antigen (see Fig. 1 a).

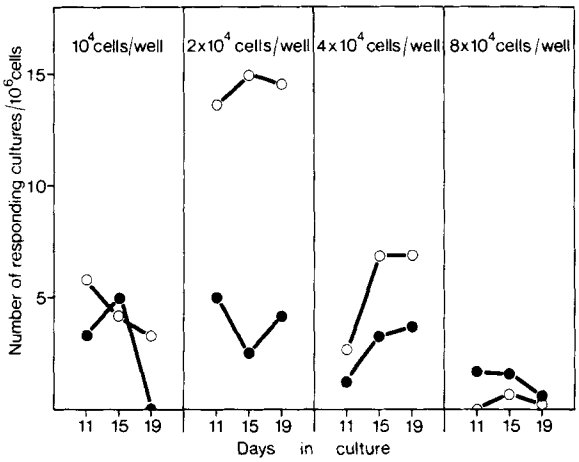

FIG. 3. Response of K6-139 PBL in microculture to Av-CHO at different cell densities. Each point is based on 120 cultures. Responses of cultures with antigen are indicated by circles and of cultures without antigen by dots. Responding cultures were identified by the spot test on Av-CHO-O-St-SRBC and SRBC. The response to SRBC was subtracted. Note that the response is optimal in a narrow range of cell densities.

As it was indicated above, in a culture system where a limiting dilution experiment cannot be performed with confidence, the frequency of precursor cells cannot be estimated accurately. For this reason we have calculated from the zero term of the Poisson distribution a frequency which we call a "frequency of responding units". Table II summarizes these "frequencies". A frequency of precursor cells of 1/48,000 (K6-139) corresponds at an average leukocyte count of 7×10^6/ml to 1.4×10^2 responding U per 1 ml blood. Thus, the rabbit, 1 yr after immunization, would have 3×10^4 precursor cells circulating in the blood (200 ml).

CLONE SIZE. The microculture system allows one to estimate both the number of precursor B cells which are involved in a response and the size of that part of clonal progeny which can be expressed as PFC (13–14).

Calculation of an average clone size is valid only if the conditions for limiting dilution are fulfilled. As these conditions could not be controlled for in the experiments reported here, the clone size estimated in this paper is essentially the expression of a "responding unit".

Microcultures containing 2×10^4 PBL per well were incubated under standard conditions in the presence of Av-CHO vaccine, and the number of anti-Av-CHO PFC was determined in each individual well.

The results of one experiment are presented in a cumulative plot shown in Fig. 4. For both days the fraction of nonresponding cultures was higher than 0.7. The median on day 11 was 23 PFC/well, while on day 13 it shifted to about 150 PFC/well. The interquartile range (± 25% around the median) is relatively narrow, especially for day 13 (45–210 PFC/well).

CLONOTYPE[2] ANALYSIS OF ANTIBODY PRODUCED IN MICROCULTURES. We have attempted an analysis of antibody secreted in vitro by subjecting supernates of single microcultures to analytical IEF. If Av-CHO-specific precursor cells are partitioned into separate microculture wells, clones silent under conventional conditions of triggering could arise which would be recognized by IEF patterns distinct from those identified in the immune serum or in the supernatants of 1-ml cultures. Supernates from 55 single microcultures that were known to contain Av-CHO-specific antibody by the spot assay were subjected to analytical IEF. Fig. 5 is an example of the kind of clonotypes observed. Of the 55 microculture supernates 18 contained products of three or more antibody-forming clones (Fig. 5, position 2). Although positive by the spot test 19 cultures were negative by IEF analysis. A summary of these data is given in Fig. 6. All clonotypes identified in the 36 IEF positive culture supernates were also present in the immune serum. Furthermore, a frequency analysis revealed that clonotypes 3, 8, and 11 (Fig. 6), predominant in the immune serum, were the most frequent ones in microcultures as well (clonotype 3 > 8 > 11).

In experiments performed with PBL of a second rabbit (K27-293) 12 pools of 10 and 14 pools of 20 microculture supernates containing anti-Av-CHO antibody by the spot test were screened for their clonotypes in comparison to the antibody found in the immune serum. It was found that each pool contained some, but not

[2] Clonotype is a monoclonal rabbit antibody recognizable by a cohesive three-band pattern with homogeneity of heavy and light chains upon sequence analysis (Braun, D. G., unpublished).

TABLE II

Frequencies of Responding Units in Rabbit PBL Primed with Av-CHO

Rabbit	Immunization courses	Av-CHO Ab*	Elapsed months‡	Day of spot assay	Minimal frequency
		mg/ml			
K31-143	1°	18	28	ND§	
	2°	23	22	ND	
	3°	14	1.5	11	1/97,000
K6-139	1°	12	29	ND	
	2°	38	10	11	1/62,000
				15–19	1/48,000
			13	18	1/33,000
K27-293	1°	31	12	17	1/74,000
	2°	15	7	20	1/58,000

* Peak levels of anti-Av-CHO antibody after primary (1°), secondary (2°), and tertiary (3°) immunization courses (5).

‡ Elapsed months postimmunization courses when bleeds were taken for isolation of PBL.

§ ND, not done.

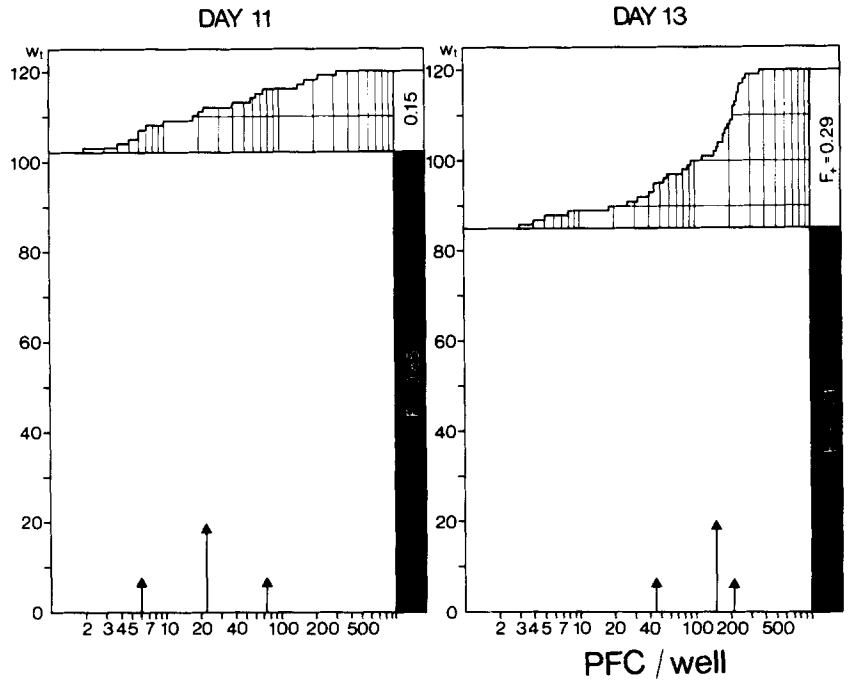

FIG. 4. Cumulative plot of in vitro responsiveness. The plot is composed of two parts. The lower zone indicates the fraction of nonresponding cultures (F_0) and the upper zone reflects the number of responding wells (F_+). The height of each "step" indicates the number of cultures with a given number of PFC. The central arrow refers to the median value where half of the responding cultures had higher PFC responses and half of them had lower responses. The interquartile ranges (enclosing 50% of the culture response around the median value) delineated by the smaller arrows give an indication of the spread of responses observed.

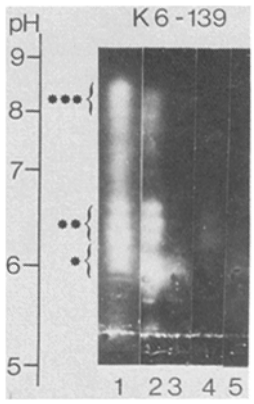

FIG. 5. IEF from single culture supernatants. Comparison of patterns was made against the antiserum obtained after a secondary (2°) course of immunization. Patterns were developed by autoradiography after binding of the [^{131}I]Av-CHO tyr. 1, 2° antiserum; 2–5 supernates from single microcultures. In position 2, a supernate with more than three clonotypes is shown. *, indicates the positions of clonotype 3; **, position of clonotype 8; and ***, position of clonotype 11 (see Fig. 7).

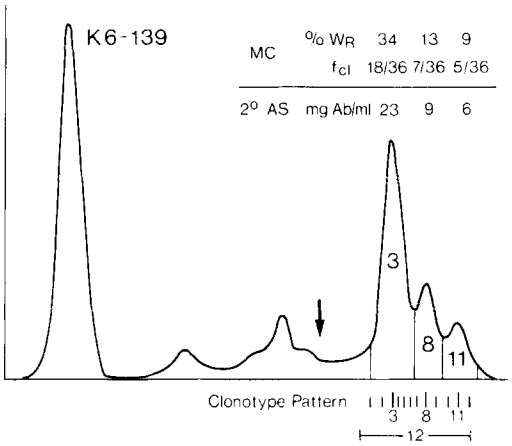

FIG. 6. Densitometric tracing of the microzone electrophoretic pattern of the 2° antiserum (2° AS) of K6-139. The arrow indicates the position of application. At the bottom a schematic diagram of the number (12) and the position of clonotypes by IEF in the 2° immune serum is given. Clonal antibodies 3, 8, and 11 dominated the in vivo response (concentration of clonotype 3 > 8 > 11). MC stands for microculture, f_{cl} for the frequency of clonotypes 3, 8, and 11 identified in microcultures; % W_R expressed the percent of reactive culture well supernates by the spot test on Av-CHO-O-St-SRBC (55 positives) that contained clonotypes 3, 8, and 11. The frequency of shared clonotype patterns in the hyperimmune serum and in microculture supernates suggests a hierarchy in the number of clonal precursor cells.

all, of the clonotypes of the immune serum (Fig. 7). The density of different clonotypes appeared to be a function of the number of cultured PBL, i.e., pools of 10 supernates harvested from cultures with 10^4 cells contained less clonotypes than pools of 10 supernates harvested from cultures with 2×10^4 cells. The total set of clonotyes identified resembled the entire clonotype pattern of the immune serum of the donor rabbit (K27-293). Again, as it was the case for PBL of K6-139,

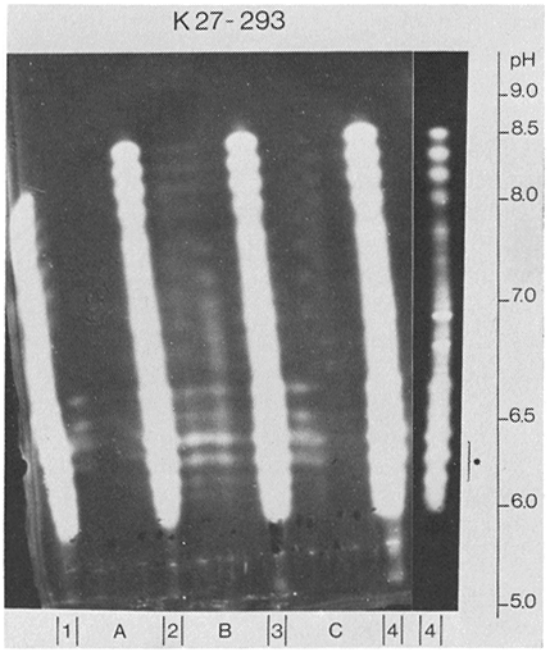

FIG. 7. Analytical IEF patterns of 2° antiserum (1–4) and of pools from microcultures supernates of K27-293 PBL. Patterns were developed by autoradiography after binding of the [^{131}I]Av-CHO tyr. (A) Six pools of supernates from 10 cultures, each containing 10^4 PBL that were cultured; (B) six pools of 20 cultures each with 2×10^4 PBL; (C) six pools of 10 cultures each with 2×10^4 PBL. * marks the position of the dominant clonotype (14.5 mg antibody/ml) in the antiserum.

the major clonotypes identified in the immune serum were expressed in microcultures more frequently than the minor ones. However, clonotypes distinct from clonal antibody in the immune serum were not found.

Discussion

It was the purpose of this study to investigate, in an in vitro system, the state of immunological memory of rabbits that had previously been hyperimmunized against streptococcal group polysaccharides. Previous work revealed a long-lived memory persisting throughout the lifetime of the rabbit at the level of specific IgG clonotypes (1) which could also be triggered in vitro (2).

Kinetics of the Response. The in vitro response of both 1-ml cultures and microcultures (10 μl) was characterized by a slow onset. This finding agrees with previous work in the SRBC (3) and the streptococcal systems (2).

It was found that the kinetics of responsiveness by identical pools of PBL (K19-205) to two different antigens (Av-CHO and A-CHO) were different. The Av-CHO response which was greater in vivo, was in three separate experiments consistently faster than the A-CHO response. It is thus possible that the amount of antibody produced by immunization is directly related to the number of memory precursor cells, and this would in turn determine the rate of the onset of a secondary in vitro response.

An in vitro response to the Av-CHO and A-CHO could be maintained for up to 42–55 days. At this time the number of recovered viable cells is 3–16% of the original cell input. After this length of time 1 out of 20 viable cells can be a specific PFC.

Rate of Antibody Production. In a previous study it was shown that de novo synthesized anti-Av-CHO antibody can be identified in culture supernates by immunoelectrophoresis and isoelectric focusing analysis (2). In the present paper the amount of antibody produced in 1-ml cultures was measured by a modified Farr technique (10). Both anti-Av-CHO and anti-A-CHO antibodies reached similar levels after 30–35 days of culture. The increase of specific anti-Av-CHO antibody by 15 μg/culture was used to estimate the secretory rate of single plaque-forming cells assuming that no antibody was enzymatically degraded and that indeed 2,000 PFC participated in antibody secretion. According to this data a single antibody-forming cell secreted: 7.5×10^{-9} g anti-Av-CHO antibody/14 days; 5.3×10^{-10} g anti-Av-CHO antibody/day; 6.15×10^{-15} g anti-Av-CHO antibody s^{-1}; 4.1×10^{-20} mol anti-Av-CHO antibody s^{-1}. Applying Avogadro's number it follows that a single antibody-forming cell stimulated in culture secreted in the order of 2.4×10^4 molecules s^{-1}. This figure agrees remarkably with the estimated secretory rate of rabbit plasma cells producing anti-SRBC antibodies (15).

The feedback effect which this accumulated antibody might have on the PFC response, either directly or through an adherent cell population[3] (16), remains undetermined within this study. To interpret the increase of antibody content in culture at a constant number of PFC is not difficult. It is, however, interesting to observe (Fig. 2) that when a certain antibody level is reached no further antibody is produced in spite of the fact that a high content of PFC is found in cultures. Whether PFC cease to secrete antibody in cultures and start again when plated remains to be shown.

Precursor Cells and Cone Size. The magnitude of the immune response of rabbits to streptococcal group polysaccharides hyperimmunized with vaccines depends on the genetic background of the rabbits (17–18). The cellular basis underlying high responsiveness is not clear. It is possible that the ability to respond with unusually high antibody levels is associated with or reflected in the number of memory cells circulating in the blood. Estimation of the number of Av-CHO specific memory cells, however, could only be expressed as responding units revealed by the microculture technique (4,13–14), because limiting dilution conditions could not be achieved by controlled titration for all cell types (B cells, T cells, and macrophages) involved in the immune reponse in vitro.

It was found that primed rabbit PBL respond optimally in a very narrow range of cell density. This optimal density was in the streptococcal system 2×10^6 PBL/ml of culture. Below and above this number the frequency of responding cultures is significantly reduced, probably because of cellular limitations at low cell densities and suppressive activities at high cell densities[3,4] (3). It was not excluded that a "crowding effect" causes suppression at high cell densities.

[3] Braun, D. G., I. Lefkovits, L. Hudson, and A. L. Luzzatti. Manuscript in preparation.

[4] Luzzati, A. L., and L. Lafleur. 1976. Suppressor cells in rabbit peripheral blood. *Eur. J. Immunol.* In press.

The frequency of precursor units found suggests that the frequency of precursor cells for the streptococcal Av-CHO in rabbit blood is 1–3 $\times$ 10^{-5} (Table II). With the same technique a frequency of 3–8 $\times$ 10^{6} was found in rabbits primed with SRBC. Although strict comparisons between these estimates cannot be made, the difference in frequency of precursor cells to the two antigens may be even greater. This is indicated by the fact that, contrary to the SRBC system 33% of the microculture supernates contained anti-Av-CHO antibody of more than one clonotype. Therefore, the actual frequency of B-cell precursors must by higher than the estimated value, because the computed precursor frequency and the size of the responding unit must be considered as underestimates. A recirculating repertoire of 3 $\times$ 10^{4} Av-CHO precursor cells with an average in vitro clone size of 200 PFC and with a secretory rate of 2 $\times$ 10^{4} antibody molecules/second per cell found here is not sufficient to explain antibody levels of 30 mg/ml, as was the case after a secondary immunization course with rabbit K6-139.

Locked-In Antibody Pattern. Estimation of the "precursor frequency" and analysis of the clonotypes triggered in vitro provided additional information concerning mechanisms that might determine clonal dominance (19). First of all, immunological memory at the IgG level of specific group polysaccharide antibodies is not only in vivo (1) but also in vitro associated with stable, i.e., locked-in antibody patterns. Secondly, product analysis by IEF of single microculture supernates permitted to estimate the precursor frequency of phenotypically dominant clonotypes expressed as responding units. In the case of rabbit K6-139 PBL the minimum frequency of Av-CHO precursor units was one precursor cell per 48,000 leukocytes in one experiment and one precursor cell per 33,000 leukocytes in a second experiment. In the case where the precursor frequency was 2 $\times$ 10^{-5} leukocytes, one Av-CHO-specific leukocyte was present among 3.5 $\times$ 10^{4} lymphocytes; this figure was derived from a lymphocyte count of 70%. This information on frequencies and the fact that the three dominant clonotypes 3, 8, and 11 were identified in 34, 13, and 9% of all anti-Av-CHO antibody-containing wells (Fig. 6) served to compute their frequencies in peripheral blood lymphocytes; according to this, cells of clonotype 3 had a frequency of 10^{-5}, those of clonotypes 8 a frequency of 4 $\times$ 10^{-6}, and those of clonotype 11 a frequency of 2.6 $\times$ 10^{-6}. A similar observation was made when the frequency of responding units and the frequency of dominant clonotypes were estimated for PBL of rabbit K27-293. Thus, when clonal dominance is established during immunization this appears to lead to a locked-in hierarchy of frequencies of clonal precursor cells persisting as circulatory memory. As a consequence such hierarchy in clonal memory is bound to determine advantages for the clonal rate of expression with any subsequent immunization. Further work will have to untangle this regulatory puzzle.

Summary

Peripheral blood lymphocytes (PBL) of rabbits previously hyperimmunized against streptococcal groups A and A-variant antigens were stimulated in vitro by the corresponding vaccines to produce group-specific antibody. This response was dependent on an optimal cell density (2 $\times$ 10^{6} cells/ml), on the presence of

antigen, it was specific and cross-reactive due to a shared rhamnose backbone of the two polysaccharide antigens, and it was highly selective, such that in a 42–55-day culture 1 out of 20 viable cells was a specific PFC. During the exponential increase of the antibody concentration at a constant number of PFC, antibodies were secreted at a rate of 2.4×10^4 molecules/s per cell until a plateau level of antibody (40 μg/culture) was reached.

The microculture system was used to determine the minimal frequency of group polysaccharide-specific precursor cells in the blood. Independent of the time elapsed since the last immunization this frequency was $1\text{–}3 \times 10^{-5}$, i.e., in the range of $1\text{–}2.8 \times 10^2$ precursor cells per ml blood. This number was further used together with the clonotype analysis of the culture supernates to calculate the frequencies of precursors of major and minor clonotypes. A hierarchy of persisting clonal memory precursor cells was found indicating that clonal dominance is determined by locked-in frequency patterns and therefore it is a phenomenon based on numbers of cells that respond to the antigen.

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