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Switch in Immunoglobulin Class Production Observed in Single Clones of Committed Lymphocytes

Abstract. *Mouse spleen cells, after stimulation with lipopolysaccharide, were cloned in culture. After 4 to 5 days, the daughter cells were stained and examined for immunoglobulin class with double immunofluorescent reagents. A switch of the stained color of these cells was observed, implying a switch from imunoglobulin M to immunoglobulin G production in the progeny of a single B cell.*

After an antigen is injected into a mouse, the first antibodies to appear in the serum are of the immunoglobulin M (IgM) class; these are followed by immunoglobulin G (IgG). This so-called switch in immunoglobulin (Ig) class expression occurs also in cultures of mitogen-stimulated B cells (bone marrow–derived lymphocytes) (*1*). Spleen cell cultures stimulated by bacterial lipopolysaccharide (LPS) contain mostly IgM-producing cells at the beginning, but a few hours after stimulation cells that produce both IgM and IgG (double producers) appear. The number of double producers increases with time; later, however, cells producing only IgG appear (*2*).

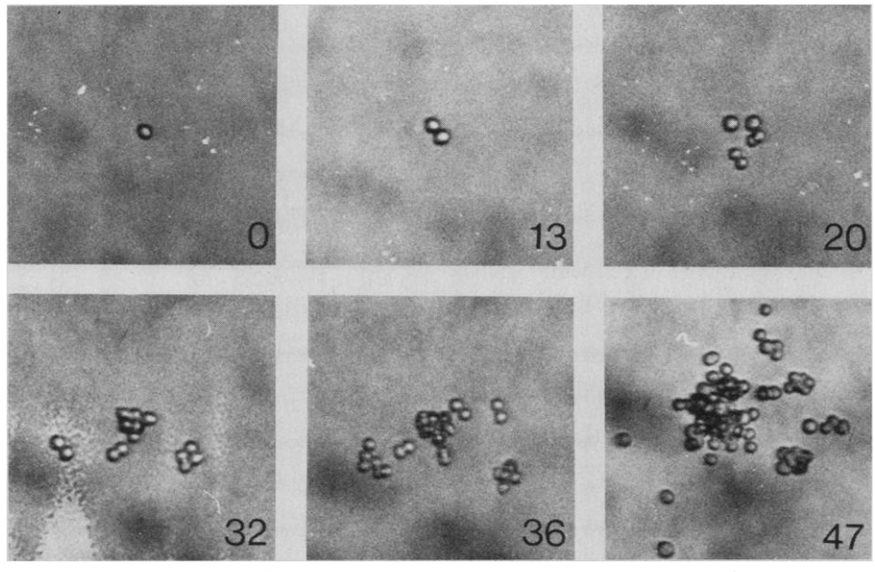
Since the presence of antiserums against the μ chain (the chain characteristic of IgM) throughout the culture period suppresses the appearance of IgG without affecting growth stimulation (*3*), it has been suggested that IgG-producing cells must derive from IgM-producing precursors. However, alternative explanations involving cooperation between cells carrying μ chains and precursors of cells carrying γ chains have not been formally excluded. We now present evidence based on single-cell experiments indicating that cells producing only IgM can give rise to daughters producing IgG.

Mouse spleen cells (2.5×10^5 cell/ml; BALB/c or C57 strain) were stimulated with LPS (20 μg/ml) in mass culture (*1*). When blast formation began, at 1 or 2 days, single blasts were withdrawn by micromanipulation and placed in Terasaki wells over a feeder layer of LPS-stimulated spleen cells in 0.3 percent agar.

Without a feeder layer, the cells divided three times at most. With a feeder layer, between 1 cell and 200 cells per well were produced 4 or 5 days after mitogenic stimulation of the single cell (Fig. 1). The shortest generation time observed was 8 to 9 hours (Figs. 1 and 2). The classes of Ig produced by 19 of the larger clones were analyzed by double immunofluorescence to detect μ- and γ-specific determinants, by a method that

Table 1. Double immunofluorescence staining for μ and γ chains. Of the 19 clones studied, five were heterogeneous. The cells were fixed for 5 minutes in ethanol before they were stained. Therefore both cytoplasmic and membrane immunoglobulins were detected. On day 4 or 5 the cells showed mainly cytoplasmic immunoglobulin. For clones 1 to 3, staining for IgG_{2a} and IgG_{2b} was not differentiated; for clones 4 and 5, only staining for IgG_{2a}, and not for IgG_{2b}, was done.

Clone	Number of cells				Remarks
	$\mu-\gamma-$	$\mu+\gamma-$	$\mu+\gamma+$	$\mu-\gamma+$	
1	5	31	3	15	IgG_2, no IgG_1
2	0	22	7	0	IgG_1, no IgG_2
3	0	28	1	0	IgG_1, no IgG_2
4	3	40	1	11	IgG_{2a}
5	1	15	8	5	IgG_{2a}



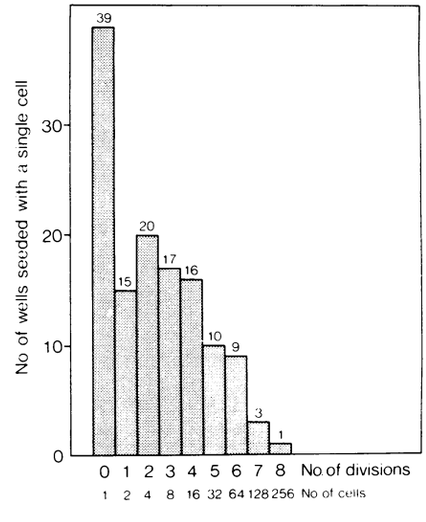


Fig. 1 (left). Clonal expansion of a single cell in culture. At day 2, after mitogenic stimulation, a blast cell was placed in a Terasaki well over a 2-day-old LPS-stimulated feeder culture. Numbers in the right lower corner represent the hours after seeding. Fig. 2 (right). Distribution of clone size. Clonal expansion of 130 single cells in culture. The cell numbers in a clone were rounded to the nearest power of 2.

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detects both membrane and cytoplasmic Ig (Table 1). Of the 19 clones, 5 were heterogeneous with respect to Ig class production.

In an experiment designed to demonstrate that heterogeneous clones can derive from a μ-bearing precursor cell, single blasts (parent cells) were allowed to divide, and one of the daughters was withdrawn. This indicator cell was stained for Ig class. The sister cell was grown to a clone, and the progeny were again stained for cytoplasmic and membrane Ig. The parent of the IgM-bearing indicator cells also yielded progeny that synthesized IgG (Fig. 3; Tables 2 and 3).

The interpretation of these experiments rests on two critical premises: the clonal nature of the cell populations that were examined and the specificity of the fluorochrome-coupled antiserums. The experimental method, coupled with direct visualization, ensures that only one cell is put into each culture well (Fig. 1). Contamination from the feeder cell layer was avoided when the Terasaki plates were prepared as follows: on day 1, 4 μl of a 0.3 percent Difco Bacto agar mass culture (7.5×10^5 cells per milliliter of feeder layer) were placed in the bottom of each well of a Terasaki plate. Then 4 μl of a 0.5 percent agar barrier layer were put on top of it and left half a day before medium containing 1 percent methylcellulose was added to completely fill the well. All layers contained RPMI 1640 medium with glutamine, supplemented with 30 m*M* Hepes buffer, 10 percent fetal calf serum, 5×10^{-5}*M* mercaptoethanol, 20 μg LPS per milliliter (*1*), and 50 units each of streptomycin and penicillin per milliliter; the medium was about 320 milliosmolar. The wells were then ready to be seeded the next day (day 2), both for the feeder cells and for the single blast cells, which were withdrawn from liquid culture. In every experiment, at least 200 culture wells were not seeded with a cell in the top layer. Control wells were rarely found to contain cells. If present, the cells were stuck at the outer part of the Terasaki well when the feeder layer was prepared and then slowly migrated into the field of vision. The experimental wells (those seeded with a single cell) were examined and those with infiltrating feeder layer cells were discarded. Even if such contamination were not detected, its incidence (less than 5/200) is too low to account for our results.

For one experimental series, the antiserum to μ chain was a rabbit antiserum to mouse MOPC-104E myeloma, made specific for μ chain by absorption with the Ig subclasses, $F(ab')_2$ fragment of IgG, and IgA of mouse. Goat antiserums to mouse IgG subclasses (Meloy Labs) were absorbed with $F(ab')_2$ fragments of normal mouse IgG (*4*). All serums were tested for specificity (*4*) by immunofluorescent double staining of the fluorochrome conjugates in all the possible combinations on mouse plasma cells in spleen smears.

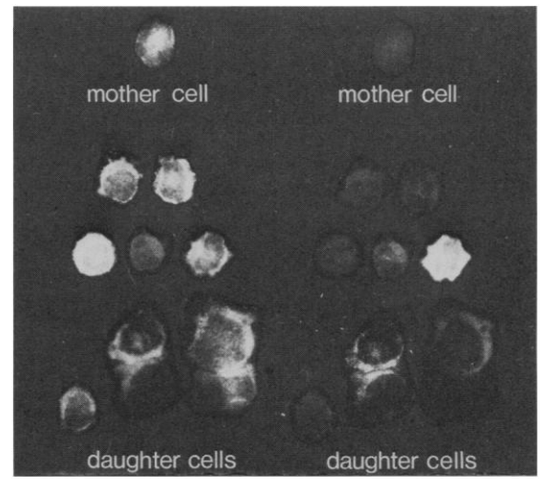

Fig. 3. Clonal expansion of a single cell, as followed by double immunofluorescence staining. Only live cells were stained. (left) Indicator cell and progeny of the sister cell stained with antibody to μ chain. (right) The identical cells stained with antibody to γ chain. The indicator cell, which was 2 days in culture, shows membrane immunoglobulins, whereas the progeny of the sister cell mainly show cytoplasmic staining. The progeny cells strongly vary in size and shape, showing asymmetry of the division process or of the differentiation process.

For another experimental series, the fluorochrome-labeled IgG to μ chains was prepared from rabbit antiserum to mouse IgM and swine antiserum to mouse IgM (Nordic, Holland) and from rabbit antiserum to mouse IgG. $F(ab')_2$ fragments were prepared from each antibody. Part was labeled with tetramethylrhodamine isothiocyanate and the rest with fluorescein isothiocyanate (*5*). The fluorescent reagents were absorbed by repeated passages over Sepharose coupled with either IgG from normal mouse serum (purified by electrophoresis) in the case of anti-μ reagents, or normal mouse serum IgM (purified by gel filtration) in the case of anti-γ reagents. The binding capacities and specificities of the multiple fractions obtained by the fluorochrome labeling were tested by immunofluorescence microscopy on fixed cells from myeloma lines producing various Ig classes [MPC 11 (K,γ_{2b}); MOPC 321 (K); MOPC 104E (λ,μ); S28 (K,γ_1); Y 5181 (K,μ); H2020 (λ,α) and MOPC 21 (P3) (K,γ_1)], as well as on normal mouse lymphocytes.

Table 2. Distribution of μ and γ chains in six mother cells.

Cells	Indicator cell	Daughter
1	$\mu-\gamma-$	$\mu-\gamma-$
2	$\mu-\gamma-$	Some $\mu+\gamma-$ daughters
3	$\mu+\gamma+$	$\mu+\gamma+$
4	$\mu+\gamma-$	$\mu+\gamma-$
5	$\mu+\gamma-$	$\mu-\gamma-, \mu+\gamma-, \mu+\gamma+$
6	$\mu+\gamma-$	$\mu-\gamma-, \mu+\gamma-,$ $\mu+\gamma+, \mu-\gamma+$

Table 3. The two clones (clones 5 and 6, shown in Table 2) showing switching to IgG_{2a}.

Clone	Number of cells			
	$\mu-\gamma-$	$\mu+\gamma-$	$\mu+\gamma+$	$\mu-\gamma+$
5	8	3	5	0
6	6	1	2	3

The implied switch in staining color in the cells cannot be explained by binding through the Fc portion of the antiserums: all reagents with antibody specificities for μ chain and γ chain were of the IgG class. If there had been binding through Fc receptors, both antiserums should have bound when the double staining procedure was used. When the $F(ab')_2$ antiserums were used, nonspecific uptake was impossible. In addition, the cells that produced IgG had IgG in the cytoplasm, as well as on the membrane. Both cytoplasmic and membrane Ig were detected because the cells were fixed for 5 minutes in ethanol before staining. Passively adsorbed Ig from the bottom feeder layer cannot account for the observed cytoplasmic staining.

In conclusion, we have presented evidence for a switch from IgM to IgG production in clones from single B cells. The cell from which the clone was derived was "committed" (having expressed its antibody specificity), if we assume that the cells yielded by the first division were alike in terms of Ig class expressed. We have verified this assumption for 40 first division cell doublets.

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References and Notes

1. J. F. Kearney and A. R. Lawton, *J. Immunol.* **115**, 671 (1975).
2. B. Pernis, L. Forni, A. Luzzati, *Cold Spring Harbor Symp. Quant. Biol.*, **41**, 175 (1976).
3. J. Anderson, W. W. Bullock, F. Melchers, *Eur. J. Immunol.* **4**, 715 (1974); J. F. Kearney, M. D. Cooper, A. R. Lawton, *J. Immunol.* **116**, 1664 (1976).
4. B. Pernis, L. Forni, K. L. Knight, in *Membrane Receptors of Lymphocytes*, M. Seligmann, J. L. Preud'homme, F. M. Kourilsky, Eds. (North-Holland, Amsterdam, 1975), p. 57.
5. F. Loor and G. E. Roelants, *Ann. N.Y. Acad. Sci.* **254**, 226 (1975).
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Direct Demonstration and Quantitation of the First Complement Component in Human Serum

Abstract. *The first component of complement, C1, can be demonstrated and quantitated in normal and pathological human serums by simple immunochemical techniques. All of the C1q, C1r, and C1s detected in normal serum was found to be in the C1 complex. A simple modification of these methods permitted the quantitation of free C1s in the presence of macromolecular C1, a technique which may prove useful in screening pathological serums.*

Earlier studies have indicated that the activity of the first component of complement, C1, requires the simultaneous presence of three proteins termed C1q, C1r, and C1s (*1–4*). Analyses of human serums by ultracentrifugation in sucrose density gradients have shown that these three proteins migrate together in a peak possessing C1 activity which is heavier than any of the individual subunits (*5*). Similar results were obtained on analyzing C1q, C1r, and C1s in normal serum by other techniques (*6*). Mixtures of isolated C1q, C1r, and C1s behaved similarly (*7, 8*). These data indicate that C1 consists of a complex of C1q, C1r, and C1s. It has not been possible, however, to directly demonstrate the C1 complex in human serum, nor has it been feasible to quantitate C1 except by activity assays requiring specialized reagents. Furthermore, it is not clear whether there are significant concentrations of free C1 subunits in normal human serum. We show here that the C1 complex can be directly demonstrated in human serum by immunochemical techniques. Minor modifications of these techniques permit quantitation of the C1 complex as well as free subunits in normal and pathological human serums.

Methods employed for the purification of C1q, C1r, and C1s and for the characterization of monospecific antiserums to each have been published (*2–4*). Titrations of the hemolytic activity of C1 in human serum were carried out as described (*7*). Normal human serum was obtained from healthy donors and used when fresh or frozen as specified in the text. Immunodiffusion analyses were performed in agarose in a buffer containing either calcium or ethylenediaminetetraacetic acid (EDTA) and at an ionic strength of 0.6 times the physiological strength, that is, conductance 8.4 mmho/cm. Single radial immunodiffusion studies were performed in agarose containing an appropriate amount of antibody to one or more of the C1 subunits. All immunodiffusion studies described in this report were carried out for 48 hours at 4°C. Diffusion in the cold was essential because C1 became activated and produced different patterns when the studies were performed at 22°C (*9*).

Antiserums to C1q, C1r, and C1s gave a single continuous fused precipitin line when diffused at 4°C against fresh normal human serum in immunodiffusion plates containing calcium (Fig. 1A). This pattern, which indicates that the C1q, C1r, and C1s antigens are on the same molecule, was observed with all normal human serums examined. The C1 complex demonstrated in this manner was not altered by repeated freezing and thawing of serum, and it was also observed in serum incubated for 1 hour at 37°C, stored for a week at 4°C, or frozen for a year at −20°C. A strikingly different pattern was observed when immunodiffusion analyses were performed in the presence of EDTA (Fig. 1B). Under these conditions, the C1 complex completely dissociated as shown by the crossing precipitin lines obtained with the various antiserums. This also indicates that the antiserums normally detect non-cross-reacting antigens.

The above studies indicated that the C1 complex could be detected in human serum by immunodiffusion. This result enabled us to employ single radial immunodiffusion to quantitate serum C1. For these studies, approximately equivalent amounts of antiserums to C1q, C1r, and C1s were incorporated into the agarose. The single ring of precipitation observed (Fig. 1C), which is the equivalent of the continuous line observed in the above Ouchterlony studies, represents C1. In accord with this interpretation, multiple precipitin rings were observed when the serums were analyzed in the presence of EDTA (Fig. 1D).

Eight normal serums and two normal serum pools were examined for their C1 content by single radial immunodiffusion. For purposes of quantitation

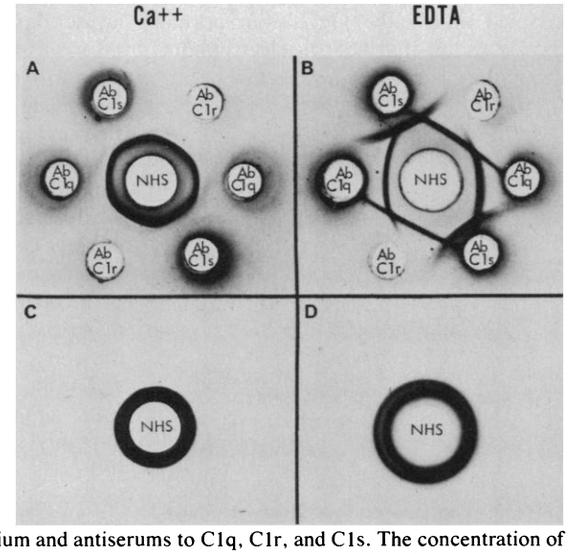

Fig. 1. Immunodiffusion analyses of C1 in normal human serum (*NHS*). Analyses were carried out in agarose containing barbital-buffered saline, *p*H 7.2, 0.6 times physiologic ionic strength for 48 hours at 4°C. Diffusion at reduced ionic strength helped to prevent C1 dissociation. (A) Double immunodiffusion analyses in 0.8 percent agarose containing 2.5 m*M* calcium. The single fused precipitin line indicates that C1q, C1r, and C1s are on the same molecule. (B) Double immunodiffusion analysis of serum containing 10 m*M* EDTA in 0.8 percent agarose containing 10 m*M* EDTA. Dissociation of the C1 complex is evident. (C) Single radial immunodiffusion in 1 percent agarose containing 2.5 m*M* calcium and antiserums to C1q, C1r, and C1s. The concentration of each antiserum used was chosen on the basis of its ability to give a precipitin ring with a diameter two to three times that of the well when reacted with physiological concentrations of the corresponding isolated antigen in single radial immunodiffusion analyses. The single ring of precipitation produced by the C1 complex in serum is evident. (D) Single radial immunodiffusion of serum containing 10 m*M* EDTA in 1 percent agarose containing 10 m*M* EDTA and antiserums to C1q, C1r, and C1s. The same concentrations of antiserums as in (C) were used. Multiple precipitin rings indicating dissociation of C1 are evident.

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